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Distribution of Binding Sequences for the Mitochondrial Import Receptors Tom20, Tom22, and Tom70 in a Presequence-carrying Preprotein and a Non-cleavable Preprotein*

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Preproteins destined for mitochondria either are synthesized with amino-terminal signal sequences, termed presequences, or possess internal targeting information within the protein. The preprotein translocase of the outer mitochondrial membrane (designated Tom) contains specific import receptors. The cytosolic domains of three import receptors, Tom20, Tom22, and Tom70, have been shown to interact with preproteins. Little is known about the internal targeting information in preproteins and the distribution of binding sequences for the three import receptors. We have studied the binding of the purified cytosolic domains of Tom20, Tom22, and Tom70 to cellulose-bound peptide scans derived from a presequence-carrying cleavable preprotein, cytochrome *c* oxidase subunit IV, and a non-cleavable preprotein with internal targeting information, the phosphate carrier. All three receptor domains are able to bind efficiently to linear 13-mer peptides, yet with different specificity. Tom20 preferentially binds to presequence segments of subunit IV. Tom22 binds to segments corresponding to the carboxyl-terminal part of the presequence and the amino-terminal part of the mature protein. Tom70 does not bind efficiently to any region of subunit IV. In contrast, Tom70 and Tom20 bind to multiple segments within the phosphate carrier, yet the amino-terminal region is excluded. Both charged and uncharged peptides derived from the phosphate carrier show specific binding properties for Tom70 and Tom20, indicating that charge is not a critical determinant of internal targeting sequences. This feature contrasts with the crucial role of positively charged amino acids in presequences. Our results demonstrate that linear peptide segments of preproteins can serve as binding sites for all three receptors with differential specificity and imply different mechanisms for translocation of cleavable and non-cleavable preproteins.

Many mitochondrial preproteins are synthesized with amino-terminal extensions, termed presequences, that function as targeting signals to direct the preproteins into mitochondria (1–3). Upon import into the mitochondrial matrix, the positively charged amphipathic presequences are cleaved off by a specific peptidase. Studies with fusion proteins consisting of a presequence and a non-mitochondrial passenger protein demonstrated that a presequence contains sufficient information for specifically directing the import of proteins into mitochondria (4–6). Extensive studies with fusion proteins and mutagenesis of presequences revealed a predominant role of presequences in targeting of cleavable preproteins (summarized in Refs. 7–9), although a contribution of the mature protein parts was also found with some preproteins (10–12).

Numerous mitochondrial preproteins, however, are synthesized as mature-sized non-cleavable preproteins. In a few cases, such preproteins were shown to contain a positively charged amphipathic targeting signal in the amino-terminal region, comparable to a non-cleaved presequence (13–17), or at an internal position of the protein (18). In many other cases, in particular membrane proteins, the available information on the targeting signals is limited. The largest family of mitochondrial membrane proteins is the inner membrane metabolite carriers, including the ADP/ATP carrier and the phosphate carrier (19–21). Each carrier protein is ~300 amino acids in length and contains six membrane-spanning segments. Three homologous domains, each with two membrane-spanning segments, constitute a carrier protein. On the one hand, the amino-terminal third of a carrier was sufficient for targeting to mitochondria, and a region in the second half of this domain seemed to constitute at least part of an import signal (22–25). On the other hand, the carboxyl-terminal two-thirds of a carrier were shown to be specifically imported into mitochondria (23, 26), suggesting the possible presence of two targeting signals in carrier preproteins.

Although a further analysis of internal targeting signals in the carrier preproteins has not been reported in the past 10 years, major progress was made in the identification and characterization of the mitochondrial outer membrane proteins that recognize and translocate preproteins. A multisubunit machinery containing at least eight different subunits was identified and termed the translocase of the outer membrane (designated Tom) (reviewed in Refs. 1–3 and 27). Three Tom proteins, Tom20, Tom22, and Tom70, were shown to function as import receptors *in vivo* and *in organello*. The expressed cytosolic domains of the import receptors were able to specifically bind mitochondrial preproteins *in vitro* (28–32). Tom22 binds cleavable preproteins; Tom70 preferentially binds non-cleavable preproteins; and Tom20 binds both cleavable and non-cleavable preproteins. Synthetic presequence peptides were able to interact with the purified cytosolic domains of Tom20 and Tom22 as shown by their competition of binding of full-length cleavable preproteins (29, 30), suggesting that the linear peptides contained sufficient information for specific recognition by the mitochondrial presequence receptors. The

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presequence peptides, however, did not influence the binding of a carrier preprotein to Tom70 or Tom20 (29).

For this study, we attempted to obtain information about the mechanism of interaction of carrier preproteins with import receptors. In particular, we asked if a carrier preprotein contained short binding sequences for receptors. Because the current knowledge did not allow the prediction of possible binding sequences in a carrier preprotein, we decided to undertake a systematic approach with cellulose-bound peptide scans (33, 34) and the expressed receptor domains. A peptide scan derived from a cleavable preprotein was selected for comparison and validated the specificity of the approach, *i.e.* Tom20 and Tom22 preferentially bound to peptides derived from the amino-terminal region of a presequence-containing preprotein. In contrast, the amino-terminal region of the phosphate carrier was found to be devoid of linear binding sequences for import receptors, yet multiple binding sequences for Tom70 and also Tom20 were observed throughout the remainder of the protein. Thus, both cleavable and non-cleavable preproteins contain linear binding sequences that are, however, of striking difference in the selection of receptors and the distribution over the preproteins. This study implies the existence of different mechanisms for recognition of non-cleavable and cleavable mitochondrial preproteins.

MATERIALS AND METHODS

Expression and Purification of the Cytosolic Tom Domains in Escherichia coli—Expression and purification were performed essentially as described (29). Briefly, the *E. coli* strain BL21(DE3) was transformed with plasmid pET19b-yTom20$_{cd}$-His$_{10}$, pET19b-yTom22$_{cd}$-His$_{10}$, or pET19b-yTom70$_{cd}$-His$_{10}$. Expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside. The bacterial pellet was resuspended in 5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9, and 2 mM phenylmethylsulfonyl fluoride, and sonified. After a clarifying spin, the supernatant was applied to Mobicol columns containing Ni^{2+}-NTA[1]-agarose resin (QIAGEN Inc.). After five washes with 80 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9, the Tom domains were eluted with 1 M imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9, and dialyzed against 10 mM MOPS, pH 7.2. One aliquot was taken to test for purity by SDS-polyacrylamide gel electrophoresis and for protein concentration by the Bradford assay or absorption at 280 nm.

Screening of Cellulose-bound Peptide Scans for Binding of Tom Proteins—The cellulose-bound peptide scans were prepared by automated spot synthesis (33, 35–37). The peptides were C-terminally linked to a cellulose membrane (Whatman) via a (β-Ala)$_2$ spacer. The dry membranes were incubated once with methanol and three times with wash buffer (100 mM KCl and 30 mM Tris-HCl, pH 7.6) at room temperature for 10 min. For analysis of protein binding activity, the membrane was incubated with 150 nM Tom protein (expressed cytosolic domains) in binding buffer (100 mM KCl, 5% (w/v) sucrose, 0.05% (v/v) Tween 20, 0.05% (w/v) bovine serum albumin, and 30 mM Tris-HCl, pH 7.6) for 60 min at 25 °C with gentle shaking. Nonspecifically bound protein was removed by washing the membrane with wash buffer for 3 min at room temperature, and peptide-bound protein was electrotransferred onto polyvinylidene difluoride membranes using a semidry blotter. The polyvinylidene difluoride membrane and the cellulose membrane were laid on top of filter papers soaked in cathode buffer (75 mM Tris base, 120 mM 6-aminohexanoic acid, and 0.01% SDS) and overlaid with filter papers soaked in anode buffers AI and AII (AI = 90 mM Tris base; and AII = 300 mM Tris base) at 4 °C. Blotting was performed at a constant power of 0.8 mA/cm^2 of cellulose membrane. The Tom protein transferred to the polyvinylidene difluoride membranes was detected by specific antisera using a fluorescence blotting substrate (ECF, Amersham Pharmacia Biotech) and a fluorescence scanning system (Fuji). Quantitation was performed with the program TINA (Raytest), including subtraction of the local background for each peptide spot. The means of at least three independent experiments for each peptide spot were used (average units). The values for different membranes were adjusted by use of identical reference peptides on each membrane. To correlate the peptide binding values with the amino acid sequence, the average units obtained for each peptide covering a given amino acid were added and divided by the number of peptides (typically four to five different peptides/residue), yielding normalized units.

Synthesis of the Phosphate Carrier and Binding to Tom70$_{cd}$ and Tom20$_{cd}$—The preprotein of the phosphate carrier was synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine/cysteine and incubated with Ni^{2+}-NTA resin-bound Tom70$_{cd}$ or Tom20$_{cd}$ for 40 min at 30 °C as described (29). Where indicated, synthetic peptides, blocked amino-terminally by acetylation and carboxyl-terminally by amidation, were added. Bound proteins were eluted with 1 M imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9, and analyzed by digital autoradiography (phosphor storage imaging technology, Molecular Dynamics, Inc.).

RESULTS AND DISCUSSION

The Cytosolic Domain of Tom20 Binds to Peptides Derived from Both the Cleavable Preprotein Cytochrome c Oxidase Subunit IV and the Non-cleavable Preprotein Phosphate Carrier—The import receptor Tom20 possesses a hydrophobic membrane anchor directly at the amino terminus, whereas the remainder of the protein is exposed to the cytosol (Fig. 1*A*) (38–40). The membrane anchor of *Saccharomyces cerevisiae* Tom20 was replaced by a His$_{10}$ tag; the resulting cytosolic domain (Tom20$_{cd}$) was expressed in *E. coli* cells, purified by Ni^{2+}-NTA affinity chromatography (29), and used in the following binding studies.

For a peptide scan derived from a cleavable mitochondrial preprotein, we chose the preprotein of subunit IV of *S. cerevisiae* cytochrome *c* oxidase (CoxIV), which has been intensively studied for *in organello* import studies (4, 6, 41–45). The scans consisted of 13-mer peptides overlapping by 10 residues, which were covalently attached to the cellulose membrane. Thus, 49 peptides were synthesized for this 155-residue preprotein. Tom20$_{cd}$ was incubated to equilibrium with the cellulose-bound peptides, followed by electrotransfer and immunodetection of the receptor domain (Fig. 1*B*). Bovine serum albumin, in a 50-fold molar excess over the receptor domains, was included in the binding assay to minimize nonspecific binding of proteins to the membranes. A quantitative analysis was performed using the TINA program, which corrects for the local background around each individual peptide spot. The best binding efficiency of Tom20$_{cd}$ was observed with peptides derived from the amino-terminal region of CoxIV (Fig. 1*D*, *upper panel*).

For a non-cleavable preprotein, the *S. cerevisiae* phosphate carrier was selected, which was previously shown to bind to Tom70 and Tom20 *in organello* and *in vitro* (29, 46). 101 peptides (13-mer), again overlapping by 10 residues, were prepared on the cellulose for this 311-residue preprotein. Tom20$_{cd}$ bound to multiple regions of the phosphate carrier peptide scan (Fig. 1*C*). By inclusion of reference peptides on each peptide scan (data not shown), the results from the CoxIV and phosphate carrier peptide scans were quantitatively comparable. The quantitation is presented in Fig. 1*D* (*lower panel*). The binding efficiency of Tom20$_{cd}$ with numerous peptides derived from the phosphate carrier was at least as high as that with amino-terminal CoxIV peptides. Interestingly, peptides derived from the amino-terminal region of the phosphate carrier did not interact with Tom20$_{cd}$.

The Cytosolic Domain of Tom22 Preferentially Binds to Peptides Derived from CoxIV—The single membrane anchor of Tom22 is located in the carboxyl-terminal half of the receptor. In contrast to Tom20 and Tom70, Tom22 exposes its amino terminus to the cytosol (Fig. 2*A*) (47–49). The cytosolic domain of *S. cerevisiae* Tom22 with a carboxyl-terminal His$_{10}$ tag (29) was expressed in *E. coli* and purified via Ni^{2+}-NTA.

Tom22$_{cd}$ was incubated with the CoxIV peptide scan (Fig. 2*B*) and the phosphate carrier peptide scan (Fig. 2*C*), followed by quantitative analysis (Fig. 2*D*). Tom22$_{cd}$ efficiently bound to

[1] The abbreviations used are: NTA, nitrilotriacetic acid; MOPS, 4-morpholinepropanesulfonic acid; Tom20$_{cd}$, Tom22$_{cd}$, and Tom70$_{cd}$, expressed cytosolic domains of Tom20, Tom22, and Tom70, respectively; CoxIV, cytochrome *c* oxidase subunit IV.

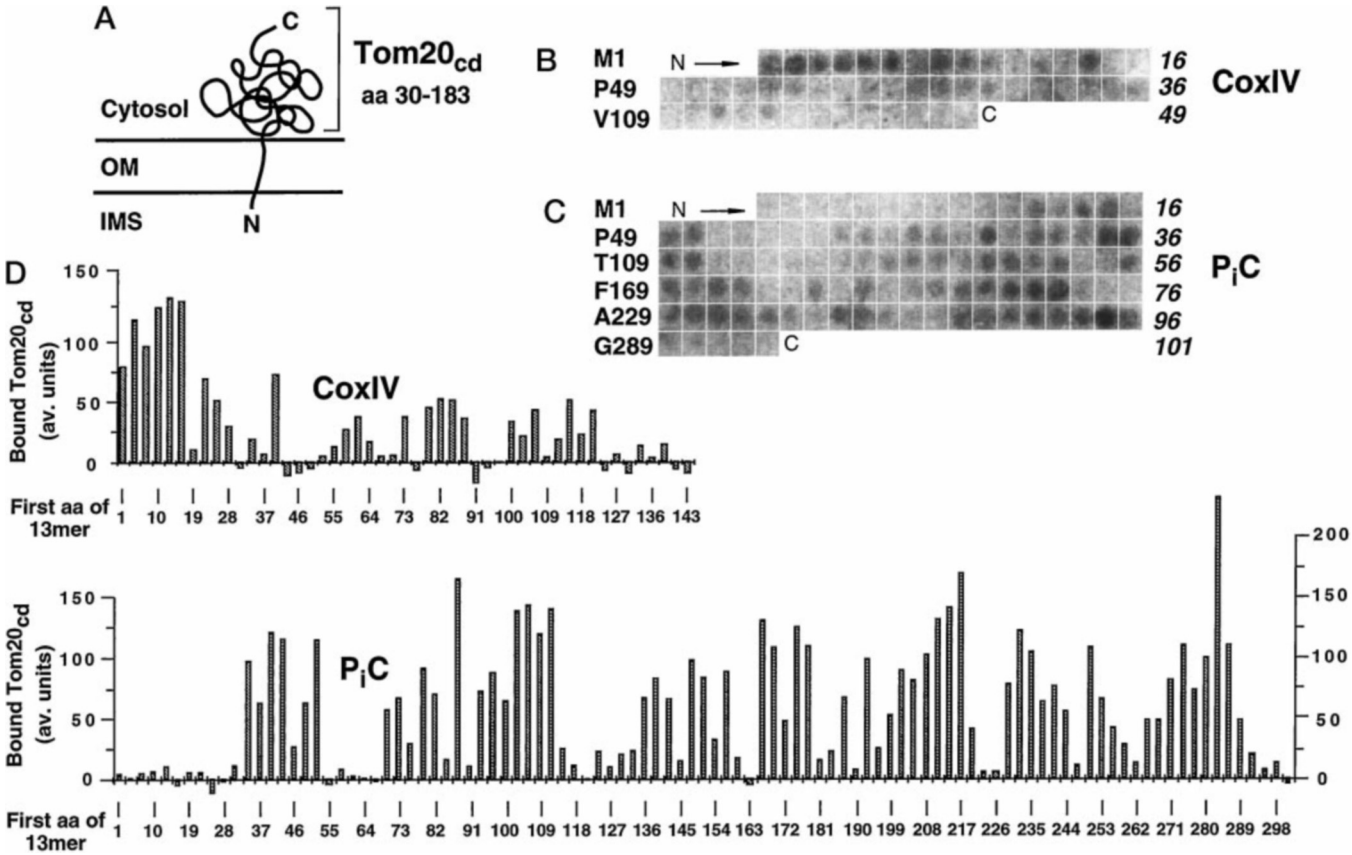

FIG. 1. **Binding of the cytosolic domain of Tom20 to peptide scans derived from cytochrome *c* oxidase subunit IV and the phosphate carrier.** *A,* the cytosolic domain of Tom20 (amino acid (*aa*) 30–183). The hydrophobic membrane anchor was replaced by an amino-terminal His_{10} tag. *OM*, outer membrane; *IMS*, intermembrane space. *B,* binding of 150 nM $Tom20_{cd}$ to a peptide scan consisting of 13-mers derived from the preprotein of *S. cerevisiae* CoxIV. The first peptide comprises amino acids 1–13 of the preprotein, the second peptide residues 4–16, and the third peptide residues 7–19, etc. The labeling on the left indicates the first amino acid of the left-most peptide of each row. The labeling on the right side indicates the number of the right-most peptide. *C*, binding of $Tom20_{cd}$ to a 13-mer peptide scan derived from the *S. cerevisiae* phosphate carrier (P_iC). Construction and labeling of the peptide scan were performed as described for *B*. *D*, quantitation of bound $Tom20_{cd}$. The amount of $Tom20_{cd}$ at each peptide spot was determined as described under "Materials and Methods" (averages from at least three independent experiments; average units).

peptide spots in the amino-terminal region of CoxIV, *i.e.* within the first 10 peptides. The peak region included peptides P4–P9 (Fig. 2, *B* and *D*, *upper panel*). In comparison, the peak region for binding of $Tom20_{cd}$ to CoxIV peptides included peptides P1–P6 (Fig. 1*D*). Thus, both $Tom20_{cd}$ and $Tom22_{cd}$ show the best binding to the amino-terminal region of CoxIV, yet the peak is shifted by approximately three peptides, *i.e.* ~9 amino acids. The remainder of the CoxIV sequence provided only few binding peptides for $Tom22_{cd}$ (Fig. 2, *B* and *D*, *upper panel*).

The phosphate carrier peptide scan revealed several binding sequences for $Tom22_{cd}$ scattered over various regions of the preprotein (Fig. 2, *C* and *D*, *lower panel*). In comparison with $Tom20_{cd}$, however, a striking difference was observed. Although numerous and efficient binding peptides for $Tom20_{cd}$ were found (Fig. 1*D*, *lower panel*), both the number of peptides and the intensity of interaction with $Tom22_{cd}$ were significantly lower.

The Cytosolic Domain of Tom70 Efficiently Binds to Peptides Derived from the Phosphate Carrier—The single membrane anchor of Tom70 is located at the amino terminus (50, 51). The membrane anchor of *S. cerevisiae* Tom70 was replaced by a His_{10} tag; the resulting cytosolic domain was expressed in *E. coli* cells and purified (29). In contrast to $Tom20_{cd}$ and $Tom22_{cd}$, binding of $Tom70_{cd}$ to the CoxIV peptide scan was nearly absent (Fig. 3*B*). A few weak binding spots were observed in the amino-terminal region (Fig. 3*D*, *upper panel*). With the phosphate carrier-derived peptide scan, however, several clusters of high efficient binding of $Tom70_{cd}$ were found (Fig. 3, *C* and *D*, *lower panel*). Interestingly, only weak binding of $Tom70_{cd}$ was observed within the first 23 peptides, comprising the amino-terminal ~70 residues of the phosphate carrier. We conclude that the non-cleavable preprotein of the phosphate carrier, but not the cleavable CoxIV preprotein, contains multiple linear binding sequences for $Tom70_{cd}$.

Comparative Analysis of the Binding Properties of Tom20, Tom22, and Tom70 at the CoxIV and Phosphate Carrier Sequences—Since the peptides overlap such that a single amino acid is covered by four to five consecutive peptides of the scan (except the penultimate amino-terminal and carboxyl-terminal residues), the quantitation data shown in Figs. 1*D*, 2*D*, and 3*D* did not readily allow a correlation of the binding efficiencies with the amino acid sequences of the preproteins. We therefore developed a score to assign the binding efficiencies for the receptor domains throughout the linear sequences of CoxIV and the phosphate carrier. For each amino acid, the binding units determined for the peptides covering this residue (average units in Figs. 1*D*, 2*D*, and 3*D*) were added and normalized for the number of peptides. The resulting normalized units can be directly aligned with the linear sequences of CoxIV (Fig. 4) and the phosphate carrier (Fig. 5). The binding properties for the CoxIV peptide scan were analyzed first since a comparison was possible to the *in organello* import studies with various constructs of CoxIV (4, 6, 41, 42, 44, 45) as well as the *in vitro* binding studies with full-length cleavable preproteins and the

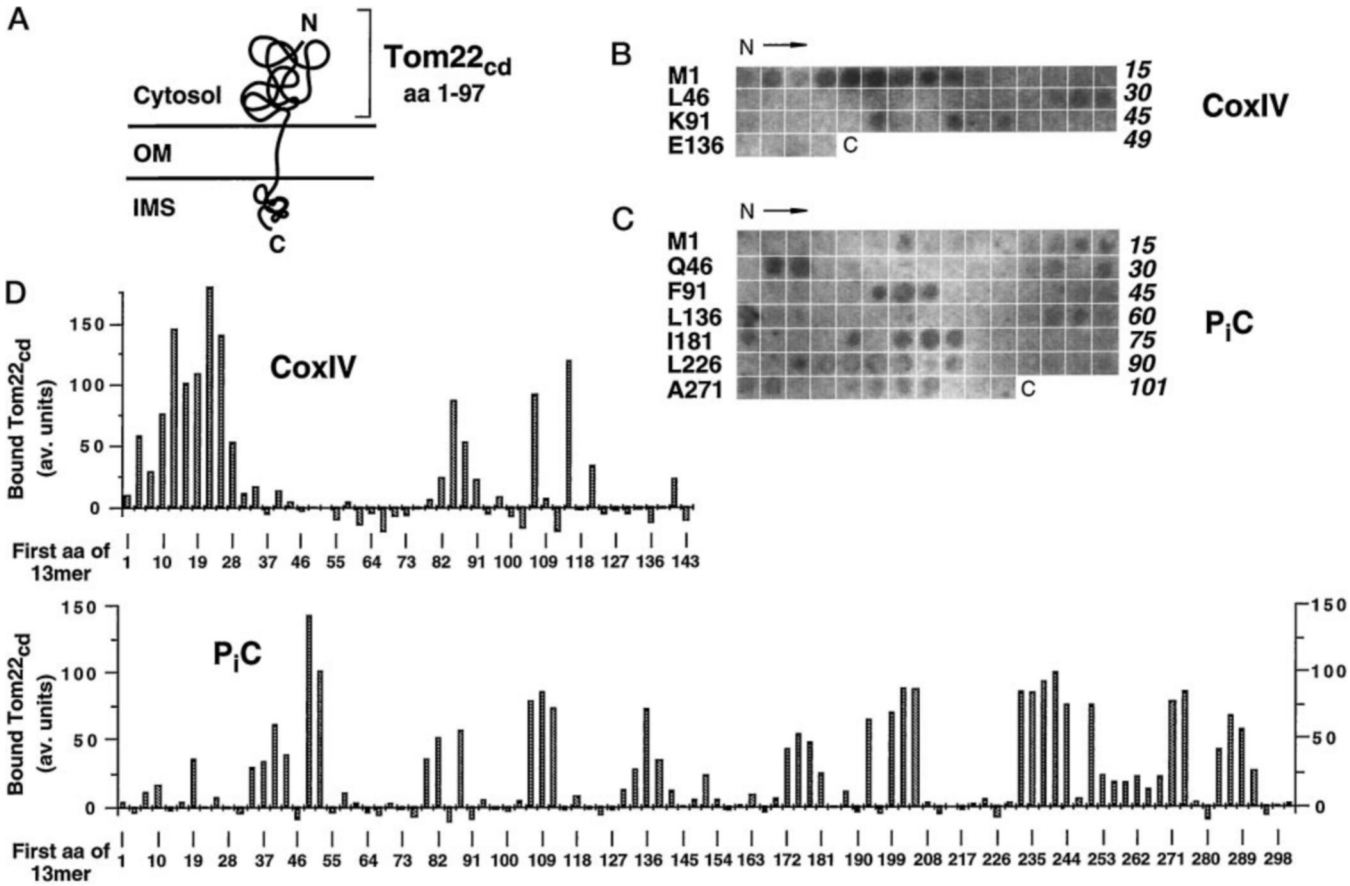

FIG. 2. **Binding of the cytosolic domain of Tom22 to peptide scans derived from CoxIV and the phosphate carrier.** *A*, the cytosolic domain of Tom22 (amino acids (*aa*) 1–97) received a carboxyl-terminal His_{10} tag. *OM*, outer membrane; *IMS*, intermembrane space. *B* and *C*, binding of 150 nM $Tom22_{cd}$ to the CoxIV-derived peptide scan and the phosphate carrier-derived peptide (P_iC) scan, respectively, was performed and analyzed as described in the legend of Fig. 1*B*. *D*, shown is the quantitation of bound $Tom22_{cd}$.

expressed cytosolic receptor domains (29, 32).

The Cleavable Preprotein of CoxIV—$Tom20_{cd}$ and $Tom22_{cd}$ strongly bound to peptides from the amino-terminal region of CoxIV, whereas $Tom70_{cd}$ bound weakly to a few amino-terminal peptides only (Fig. 4). This agrees well with the import studies in mitochondria. Import of cleavable preproteins is preferentially directed by the presequences via the Tom20-Tom22 pathway and does not require Tom70 (4, 5, 38, 40, 47, 49, 52, 53).

A comparison between receptor binding to short peptides that lack higher order structure and binding to full-length proteins with folded structure poses the problem that folding differences may influence the exposure or formation of potential binding sites. With presequences, however, this problem is minimized since several studies demonstrated that the presequences of preproteins are in loosely folded conformations (8, 54) and thus should be close to the structural properties of short peptides. Indeed, synthetic peptides derived from the presequence of CoxIV specifically competed with the binding of full-length cleavable preproteins to $Tom20_{cd}$ and $Tom22_{cd}$ (29). No interaction of presequences with $Tom70_{cd}$ was observed in the *in vitro* binding studies with various full-length preproteins. In particular, synthetic peptides derived from the CoxIV presequence did not influence the interaction of preproteins (containing internal targeting information) with $Tom70_{cd}$ (29). This indicates that the weak binding of $Tom70_{cd}$ to CoxIV peptides (up to 40 normalized units) (Fig. 4) is not of functional relevance. In contrast, the areas of the best binding of $Tom20_{cd}$ or $Tom22_{cd}$ to CoxIV peptides yielded binding scores of >100 normalized units (Fig. 4). Based on these considerations and the profiles obtained for binding to the phosphate carrier peptide scan (see below), we operationally divided binding into three categories to allow a qualitative comparison of binding intensities: "strong binding" (≥100 normalized units), "binding" (≥60 normalized units), and "poor to non-binding" (<60 normalized units).

Significant binding of $Tom20_{cd}$ was almost exclusively confined to the presequence of CoxIV (Fig. 4), which is in good agreement with the dominant role of Tom20 as a presequence receptor (29, 38, 40, 52). The peak of best binding of $Tom22_{cd}$ was shifted by ~9 amino acids toward the carboxyl terminus of CoxIV and thus was observed at the carboxyl-terminal end of the presequence and in the amino-terminal region of the mature protein part (Fig. 4). This supports the proposed role of Tom22 as a presequence receptor (29, 48, 49, 53), yet additionally explains the contributions of mature protein parts, particularly in the amino-terminal region, to the targeting efficiencies of some preproteins (10). A structural analysis of the CoxIV presequence showed the formation of a helical structure in the amino-terminal half, whereas the remainder of the presequence did not adopt a regular secondary structure (55), suggesting that the helical structure may be of particular importance for the interaction with Tom20. *In organello* import studies, together with an analysis of the structural composition of the Tom machinery, indicated that preproteins are initially recognized by Tom20 and are subsequently transferred to Tom22 before their insertion into the general import pore (47, 49, 56–58). The peptide binding properties seen here support such a model in that Tom20 initially recognizes the very first part of a preprotein. Tom22 neighboring the import pore binds

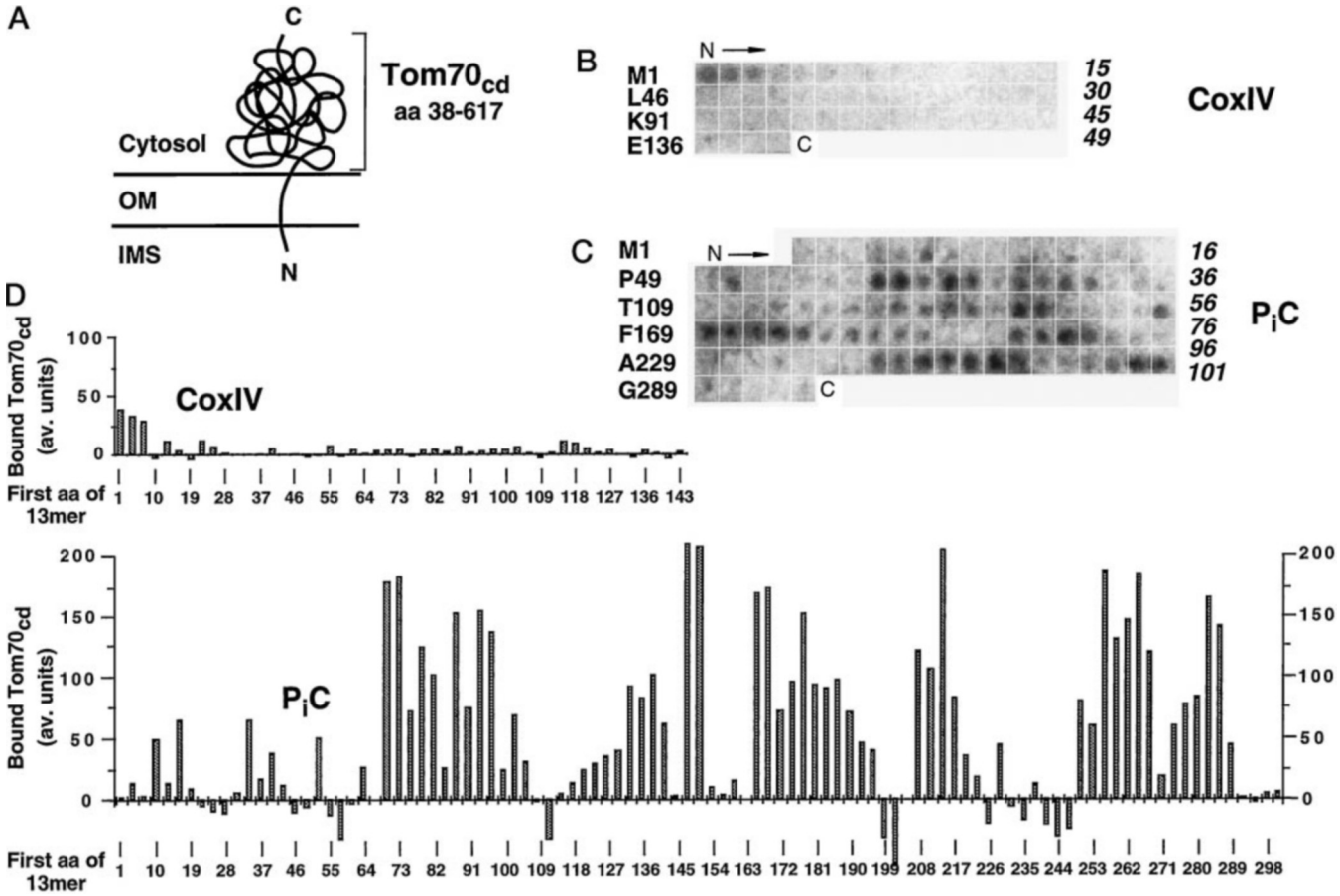

FIG. 3. **The cytosolic domain of Tom70 preferentially binds to peptides derived from the phosphate carrier, but not from CoxIV.** *A*, the cytosolic domain of Tom70 (amino acids (*aa*) 38–617) was equipped with an amino-terminal His_{10} tag instead of the membrane anchor. *OM*, outer membrane; *IMS*, intermembrane space. *B* and *C*, binding of 150 nM $Tom70_{cd}$ to the CoxIV-derived peptide scan and the phosphate carrier-derived peptide (P_iC) scans, respectively, was performed and analyzed as described in the legend of Fig. 1*B*. *D*, shown is the quantitation of bound $Tom70_{cd}$.

further toward the carboxyl terminus of the preprotein such that the amino terminus of the presequence is free for interaction with the import pore. The remainder of the CoxIV sequence yielded only few efficient binding peptides for receptor domains, particularly a potential binding of $Tom22_{cd}$ to regions in the carboxyl-terminal half of mature CoxIV (Fig. 4, *lower panel*). Future studies will have to address the significance of these possible recognition sequences.

The Non-cleavable Preprotein of the Phosphate Carrier—With $Tom70_{cd}$, at least five regions of the phosphate carrier with strong binding properties were found (Fig. 5). A strong binding region was found in the second half of each of the three carrier domains in the area of the second transmembrane segment (TM2, TM4, and TM6) and carboxyl-terminal to this. Interestingly, the remainder of the amino-terminal domain, *i.e.* the amino-terminal 70 amino acids of the phosphate carrier, was completely devoid of efficient binding sequences for Tom70 (Fig. 5, *upper panel*). The middle domain and the carboxyl-terminal domain of the phosphate carrier contain an additional strong binding region each: between the two transmembrane segments in the case of the middle domain (between TM3 and TM4) (Fig. 5, *middle panel*) and in the area of the first transmembrane segment of the carboxyl-terminal domain (TM5) (Fig. 5, *lower panel*). The binding properties of Tom70 for the carrier-derived peptide scan agree with and extend the current information on targeting signals of carrier preproteins that were obtained by *in organello* import studies with constructs of preproteins. Douglas and co-workers (22, 24, 25) showed that the amino-terminal third of the ADP/ATP carrier contained sufficient information for targeting to mitochondria, yet the amino-terminal 72 residues were dispensable, indicating the presence of targeting information in a region between residues 73 and 111. However, the carboxyl-terminal two-thirds of two carriers were imported into mitochondria in the absence of the amino-terminal domains, demonstrating the presence of at least two independent targeting signals within these preproteins (23, 26). The presence of several strong binding regions in the middle domain and the carboxyl-terminal domain with a comparable affinity compared with the region in the amino-terminal domain (Fig. 5) suggests the presence of multiple receptor-binding signals in carriers. Since the carrier constructs mentioned above were imported with lower efficiency than the full-length carrier (26), multiple signals appear to increase the efficiency of the import process. It has been speculated that the receptor function of Tom70 may include chaperone-like activities in preventing aggregation and misfolding of the membrane proteins (27). The presence of multiple binding sequences in a preprotein would facilitate such a chaperone function.

The overall binding efficiencies of Tom70 for peptides of the phosphate carrier peptide scan were higher than those of Tom20 (Fig. 5). This is in agreement with the predominant role of Tom70 in carrier import, whereas Tom20 is involved in carrier import to a smaller, yet significant degree (39, 40, 51, 59). Indeed, $Tom20_{cd}$ is able to bind carrier preproteins (29) and interacts with several regions of the phosphate carrier peptide scan (Fig. 5). The binding regions correlate in part with those of $Tom70_{cd}$; two of the three strong binders coincide with strong

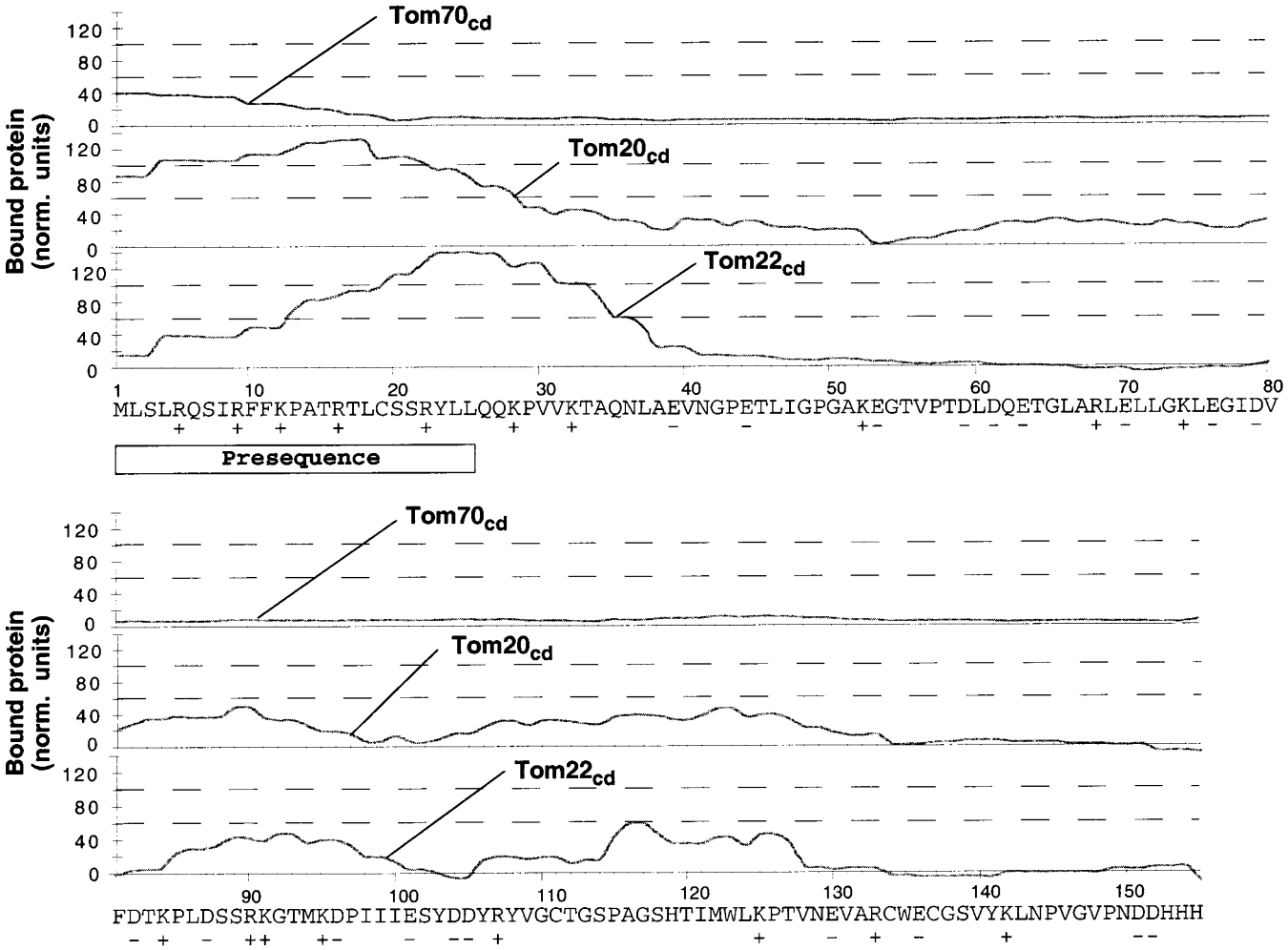

FIG. 4. **Distribution of binding sequences for Tom70, Tom20, and Tom22 on the cleavable preprotein of CoxIV.** The binding intensities of the cytosolic Tom domains for the CoxIV-derived 13-mer peptides determined in the experiments of Figs. 1–3 were converted to sequence-specific normalized (*norm.*) units as described under "Materials and Methods." The primary structure of CoxIV is indicated in single-letter code. The presequence comprises residues 1–25. The threshold values for "binders" (60 normalized units) and "strong binders" (100 normalized units) are indicated by *dashed lines*.

binders of $Tom70_{cd}$ in the carboxyl-terminal domain. Moreover, the amino-terminal 40 residues are devoid of significant binding peptides for $Tom20_{cd}$. However, there are three regions of the phosphate carrier peptide scan where binding of $Tom70_{cd}$ is absent, whereas $Tom20_{cd}$ shows significant binding: the regions between the transmembrane segments of the amino-terminal domain and the carboxyl-terminal domain and a segment around the beginning of the first transmembrane segment (TM3) of the middle domain, representing the third strong binder (Fig. 5).

Conflicting results were reported for the role of Tom22 in the import of carrier preproteins. Although translocation intermediates of carrier preproteins were shown to be transported to the import pore of intact mitochondria via Tom22 (47, 60), no binding of carrier preproteins to the expressed cytosolic domain of Tom22 was observed (29). The binding yields of $Tom22_{cd}$ with the phosphate carrier peptide scan were, overall, clearly lower than those of $Tom70_{cd}$ or $Tom20_{cd}$, but a few regions with binding scores at or above the lower threshold were observed (Fig. 5). Interestingly, the Tom22-binding regions largely coincide with the Tom20-binding regions. Since $Tom22_{cd}$ does not bind the full-length phosphate carrier synthesized in a cell-free system (29), it may be speculated that the Tom22-binding segments in the phosphate carrier are not exposed on the surface of the preprotein, but become accessible to the receptor only by unfolding of the carrier during the translocation process. It is thus possible that segments that are bound by Tom20 or Tom22, but not by Tom70, are not exposed on the surface of the preprotein, in agreement with the view that Tom70 is the first mitochondrial import receptor that interacts with carrier preproteins.

The few peptide sequences of the phosphate carrier that bind $Tom22_{cd}$ were all characterized by a positive net charge (Fig. 5), as is the case with the presequences that efficiently bind to $Tom22_{cd}$. The cytosolic domain of Tom22 contains a large abundance of negatively charged residues (47–49). Therefore, an ionic interaction between binding peptides and $Tom22_{cd}$ is likely (29).

Synthetic Peptides Derived from the Phosphate Carrier Peptide Scan Compete with Binding of the Full-length Phosphate Carrier to Tom70 and Tom20—The predominant role of presequences in import of preproteins into isolated organelles and the competition of preprotein binding to $Tom20_{cd}$ and $Tom22_{cd}$ by synthetic presequence peptides validate the main observations of the CoxIV peptide scan and thus the use of this peptide scan for defining targeting sequences in a cleavable preprotein. The information on signals in carrier preproteins and binding to the Tom70 or Tom20 receptor is more limited, yet agrees with the observations made with the phosphate carrier peptide scan. To directly test predictions made from this peptide scan, we synthesized four 13-mer peptides in soluble form (Fig. 6*A*). The addition of short peptides to *in organello* import studies is

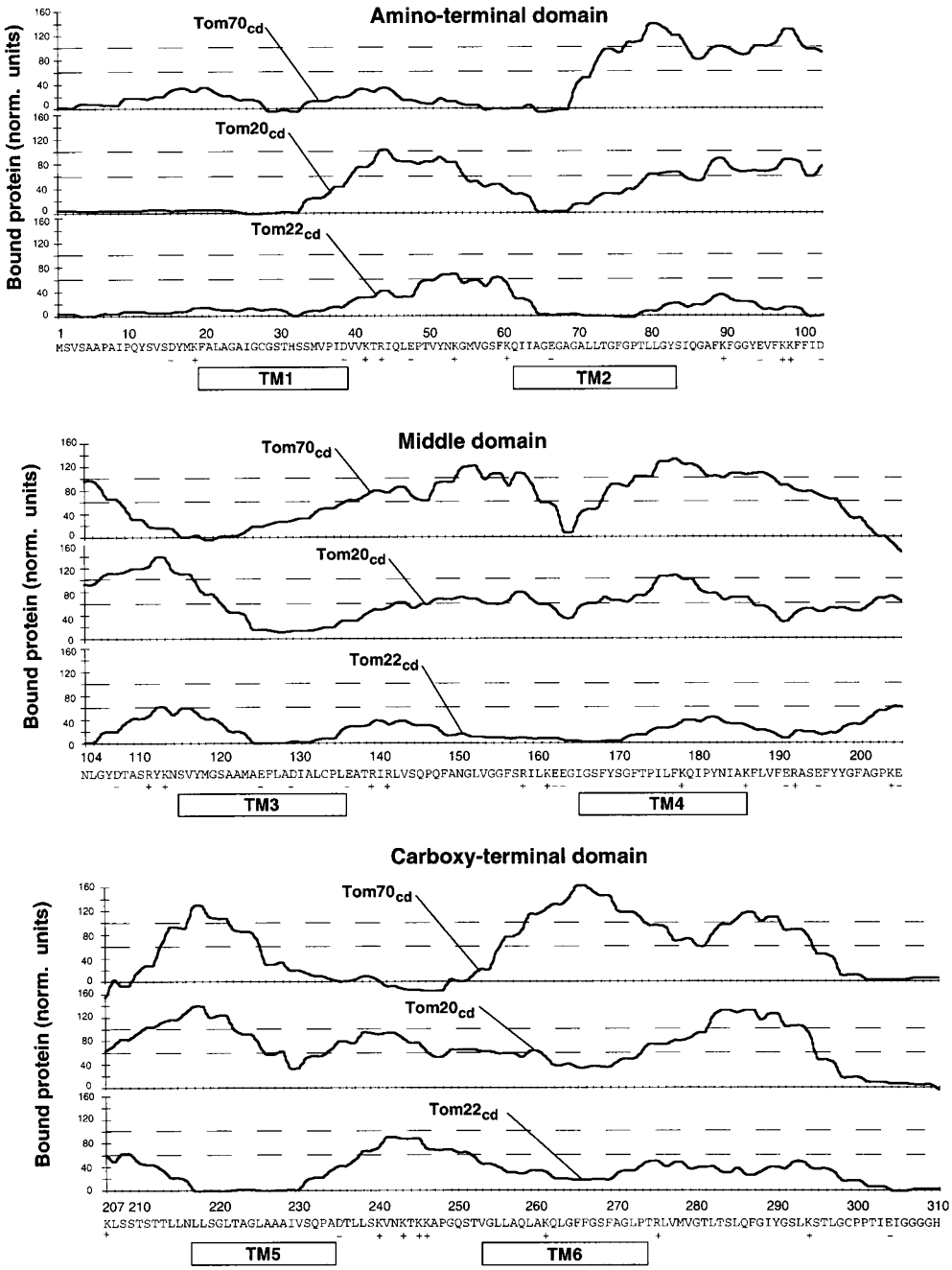

FIG. 5. **Distribution of binding sequences for Tom70, Tom20, and Tom22 on the non-cleavable preprotein phosphate carrier.** The binding intensities of the cytosolic Tom domains for the 13-mer peptides derived from the phosphate carrier were converted to normalized (*norm.*) units (directly comparable to the values for CoxIV in Fig. 4). Thresholds for binders (60 normalized units) and strong binders (100 normalized units) are indicated by *dashed lines*. The six predicted transmembrane segments (*TM*) (19) are marked.

difficult to interpret since short peptides bind to and insert into the mitochondrial membranes, thereby disturbing various mitochondrial functions (44, 45, 61, 62). We therefore tested the influence of the synthetic peptides on the binding of the full-length phosphate carrier to $Tom70_{cd}$ or $Tom20_{cd}$.

Peptides P1–P3 were selected for the binding assay with Tom70 since peptides P1 and P2 are derived from the strong Tom70-binding region of the phosphate carrier peptide scan, whereas peptide P3 is from a non-binding region. The preprotein of the phosphate carrier was synthesized and radiolabeled in rabbit reticulocyte lysate, and the interaction with Ni^{2+}-NTA resin bound $Tom70_{cd}$ was analyzed (29). Peptides P1 and P2 strongly inhibited the binding of the phosphate carrier (Fig. 6*B*, *columns 2* and *3*), whereas peptide P3 did not show an effect (*column 4*), in full agreement with the binding studies on the cellulose membrane.

For the binding assay with Tom20, peptides P2–P4 were used since, on the peptide scan, peptides P2 and P3 efficiently bound $Tom20_{cd}$, whereas P4 had a binding score below 50 units. Indeed, peptides P2 and P3 strongly inhibited the binding of the phosphate carrier to Ni^{2+}-NTA resin-bound $Tom20_{cd}$ (Fig. 6*C*, *columns 2* and *3*), whereas peptide P4 did not (*column 4*).

We conclude that peptides P1 and P2 in the case of Tom70 and peptides P2 and P3 in the case of Tom20 bind to sites of the receptors that are crucial for the interaction with the full-length preprotein. In contrast, peptide P3 (for Tom70) and peptide P4 (for Tom20) do not influence the specific receptor activity. These results provide good evidence for the validity of

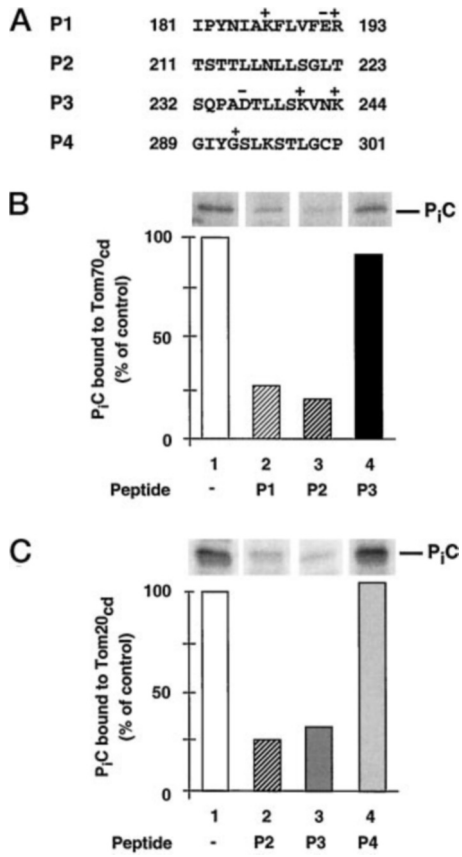

FIG. 6. **Linear sequences identified with peptide libraries inhibit binding of the full-length phosphate carrier to Tom70 and Tom20.** *A*, shown are synthetic peptides derived from the *S. cerevisiae* phosphate carrier (P_iC) sequence. The peptides were blocked amino-terminally by acetylation and carboxyl-terminally by amidation. *B*, the preprotein of the phosphate carrier was synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine/cysteine. $Tom70_{cd}$ was bound to Ni^{2+}-NTA resin and incubated with the phosphate carrier preprotein in the presence of peptide P1, P2, or P3 (200 μM each) as described under "Materials and Methods" (29). Bound phosphate carrier was quantified by digital autoradiography. The amount of phosphate carrier bound to $Tom70_{cd}$ in the absence of peptide was set to 100% (control). *C*, the experiment was performed as described for *B*, except that $Tom20_{cd}$ and peptides P2–P4 were used (200 μM peptides P2 and P4 and 100 μM peptide P3).

the phosphate carrier-derived peptide scan and indicate that the specific binding site of each receptor is able to recognize different peptides.

Positively charged residues and an amphipathic character are crucial for the function of mitochondrial presequences (8, 63–65). Moreover, Káldi *et al.* (66) reported that positively charged internal segments in Tim proteins function as targeting signals. However, Davis *et al.* (67) showed that targeting signals are not located in these positively charged segments, but in at least two of the predicted four hydrophobic segments of Tim23. Although an assessment of the predicted hydrophobic character of the phosphate carrier indicated a loose preference for more hydrophobic segments among the strong Tom70 binders, the charge distribution of the phosphate carrier peptides clearly does not account for the efficiency of interaction with Tom70 or Tom20. With both receptors, an uncharged peptide (P2) and a charged peptide (P1 for Tom70; P3 for Tom20; and both with 2 positively charged residues and 1 negatively charged residue) show specific binding properties. Interestingly, peptide P3 is a binder for Tom20, but a non-binder for Tom70, supporting the view of a different specificity of the binding sites of the two receptors. Together with the finding that presequence peptides do not influence the binding of the phosphate carrier to Tom70 or Tom20 (29), we conclude that the mechanism of interaction of phosphate carrier peptides with receptors is significantly different from that of presequence peptides. These observations support the proposal that Tom20 contains two distinct binding sites, one for presequence-carrying preproteins and one for preproteins with internal targeting information (29, 32, 68).

Conclusions—We report the first systematic analysis of receptor-binding sequences in mitochondrial preproteins. Short peptides derived from cleavable or non-cleavable preproteins can function as specific binding sequences for the import receptors Tom20, Tom22, and Tom70. The use of peptide scans for identification of mitochondrial targeting signals is validated by the good agreement of our conclusions with the *in organello* import studies and *in vitro* binding studies using entire cleavable preproteins. The nature of targeting signals in a carrier preprotein has been unknown. We show that multiple recognition sequences for import receptors are present and confirm this conclusion by a specific inhibition of the interaction between import receptors and a full-length carrier by short peptides. It cannot be formally excluded that, in addition to linear recognition sequences, more complex, discontinuous targeting information is present in preproteins. However, the binding specificities for different receptors and preproteins determined with the peptide scans reflect very well the specificities that are observed by importing full-length preproteins into mitochondria.

In a typical cleavable preprotein, the binding sequences are largely confined to the amino-terminal presequence and the adjacent portion of the mature protein, whereas binding peptides derived from a carrier preprotein are found in several clusters throughout the protein, except for the lack of binding sequences in the amino-terminal region. Cleavable preproteins enter mitochondria with their amino terminus first (69, 70). It may be speculated that carrier preproteins are bound by the import receptors at multiple binding sites and enter the import machinery with non-amino-terminal portions first. Presequences are characterized by an abundance of positively charged residues. With the phosphate carrier peptide scan, charged amino acids are apparently not the determinant of the targeting function of peptides that specifically bind Tom70 or Tom20 since both charged and uncharged peptides can be effective. These results suggest a fundamental difference in the mechanism of targeting of cleavable preproteins and carrier preproteins.

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REFERENCES

1. Schatz, G., and Dobberstein, B. (1996) *Science* **271,** 1519–1526
2. Neupert, W. (1997) *Annu. Rev. Biochem.* **66,** 863–917
3. Pfanner, N., Craig, E. A., and Hönlinger, A. (1997) *Annu. Rev. Cell Dev. Biol.* **13,** 25–51
4. Hurt, E. C., Pesold-Hurt, B., and Schatz, G. (1984) *FEBS Lett.* **178,** 306–310
5. Horwich, A. L., Kalousek, F., Mellmann, I., and Rosenberg, L. E. (1985) *EMBO J.* **4,** 1129–1135
6. Hurt, E. C., Pesold-Hurt, B., Suda, K., Oppliger, W., and Schatz, G. (1985) *EMBO J.* **4,** 2061–2068
7. Hurt, E. C., and van Loon, A. P. G. M. (1986) *Trends Biochem. Sci.* **11,** 204–207
8. Roise, D., and Schatz, G. (1988) *J. Biol. Chem.* **263,** 4509–4511
9. Horwich, A. (1990) *Curr. Opin. Cell Biol.* **2,** 625–633
10. Bedwell, D. M., Klionsky, D. J., and Emr, S. D. (1987) *Mol. Cell. Biol.* **7,** 4038–4047
11. Pfanner, N., Müller, H. K., Harmey, M. A., and Neupert, W. (1987) *EMBO J.* **6,** 3449–3454
12. Arnold, I., Fölsch, H., Neupert, W., and Stuart, R. A. (1998) *J. Biol. Chem.* **273,** 1469–1476
13. Hurt, E. C., Müller, U., and Schatz, G. (1985) *EMBO J.* **4,** 3509–3518
14. Arakawa, H., Amaya, Y., and Mori, M. (1990) *J. Biochem. (Tokyo)* **107,** 160–164
15. Rospert, S., Junne, T., Glick, B. S., and Schatz, G. (1993) *FEBS Lett.* **335,** 358–360
16. Hahne, K., Haucke, V., Ramage, L., and Schatz, G. (1994) *Cell* **79,** 829–839
17. Jarvis, J. A., Ryan, M., Hoogenraad, N. J., Craik, D. J., and Hoj, P. B. (1995) *J. Biol. Chem.* **270,** 1323–1331

18. Fölsch, H., Guiard, B., Neupert, W., and Stuart, R. A. (1996) *EMBO J.* **15,** 479–487
19. Palmieri, F. (1994) *FEBS Lett.* **346,** 48–54
20. El Moualij, B., Duyckaerts, C., Lamotte-Brasseur, J., and Sluse, F. E. (1997) *Yeast* **13,** 573–581
21. Nelson, D. R., Felix, C. M., and Swanson, J. M. (1998) *J. Mol. Biol.* **277,** 285–308
22. Adrian, G. S., McCammon, M. T., Montgomery, D. L., and Douglas, M. G. (1986) *Mol. Cell. Biol.* **6,** 626–634
23. Liu, X., Bell, A. W., Freeman, K. B., and Shore, G. C. (1988) *J. Cell Biol.* **107,** 503–509
24. Smagula, C., and Douglas, M. G. (1988) *J. Biol. Chem.* **263,** 6783–6790
25. Smagula, C. S., and Douglas, M. G. (1988) *J. Cell. Biochem.* **36,** 323–327
26. Pfanner, N., Hoeben, P., Tropschug, M., and Neupert, W. (1987) *J. Biol. Chem.* **262,** 14851–14854
27. Ryan, M. T., and Pfanner, N. (1998) *Biol. Chem.* **379,** 289–294
28. Schlossmann, J., Dietmeier, K., Pfanner, N., and Neupert, W. (1994) *J. Biol. Chem.* **269,** 11893–11901
29. Brix, J., Dietmeier, K., and Pfanner, N. (1997) *J. Biol. Chem.* **272,** 20730–20735
30. Komiya, T., Rospert, S., Schatz, G., and Mihara, K. (1997) *EMBO J.* **16,** 4267–4275
31. Komiya, T., Rospert, S., Koehler, C., Looser, R., Schatz, G., and Mihara, K. (1998) *EMBO J.* **17,** 3886–3898
32. Schleiff, E., Shore, G. C., and Goping, I. S. (1997) *J. Biol. Chem.* **272,** 17784–17789
33. Reineke, U., Sabat, R., Kramer, A., Stigler, R. D., Seifert, M., Michel, T., Volk, H. D., and Schneider-Mergener, J. (1996) *Mol. Divers.* **1,** 141–148
34. Rüdiger, S., Germeroth, L., Schneider-Mergener, J., and Bukau, B. (1997) *EMBO J.* **16,** 1501–1507
35. Frank, R. (1992) *Tetrahedron* **48,** 9217–9232
36. Kramer, A., Schuster, A., Reineke, U., Malin, R., Volkmer-Engert, R., Landgraf, C., and Schneider-Mergener, J. (1994) *Methods Companion Methods Enzymol.* **6,** 388–395
37. Kramer, A., and Schneider-Mergener, J. (1998) *Methods Mol. Biol.* **87,** 25–39
38. Schneider, H., Söllner, T., Dietmeier, K., Eckerskorn, C., Lottspeich, F., Trülzsch, B., Neupert, W., and Pfanner, N. (1991) *Science* **254,** 1659–1662
39. Ramage, L., Junne, T., Hahne, K., Lithgow, T., and Schatz, G. (1993) *EMBO J.* **12,** 4115–4123
40. Moczko, M., Ehmann, B., Gärtner, F., Hönlinger, A., Schäfer, E., and Pfanner, N. (1994) *J. Biol. Chem.* **269,** 9045–9051
41. Eilers, M., and Schatz, G. (1986) *Nature* **322,** 228–232
42. Allison, D. S., and Schatz, G. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 9011–9015
43. van Loon, A. P. G. M., and Schatz, G. (1987) *EMBO J.* **6,** 2441–2448
44. Glaser, S. M., and Cumsky, M. G. (1990) *J. Biol. Chem.* **265,** 8808–8816
45. Glaser, S. M., and Cumsky, M. G. (1990) *J. Biol. Chem.* **265,** 8817–8822
46. Dietmeier, K., Zara, V., Palmisano, A., Palmieri, F., Voos, W., Schlossmann, J., Moczko, M., Kispal, G., and Pfanner, N. (1993) *J. Biol. Chem.* **268,** 25958–25964
47. Kiebler, M., Keil, P., Schneider, H., van der Klei, I. J., Pfanner, N., and Neupert, W. (1993) *Cell* **74,** 483–492
48. Lithgow, T., Junne, T., Suda, K., Gratzer, S., and Schatz, G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 11973–11977
49. Hönlinger, A., Kübrich, M., Moczko, M., Gärtner, F., Mallet, L., Bussereau, F., Eckerskorn, C., Lottspeich, F., Dietmeier, K., Jacquet, M., and Pfanner, N. (1995) *Mol. Cell. Biol.* **15,** 3382–3389
50. Hase, T., Riezman, H., Suda, K., and Schatz, G. (1983) *EMBO J.* **2,** 2169–2172
51. Steger, H. F., Söllner, T., Kiebler, M., Dietmeier, K. A., Pfaller, R., Trülzsch, K. S., Tropschug, M., Neupert, W., and Pfanner, N. (1990) *J. Cell Biol.* **111,** 2353–2363
52. Söllner, T., Griffiths, G., Pfaller, R., Pfanner, N., and Neupert, W. (1989) *Cell* **59,** 1061–1070
53. Mayer, A., Nargang, F. E., Neupert, W., and Lill, R. (1995) *EMBO J.* **14,** 4204–4211
54. Schmid, D., Jaussi, R., and Christen, P. (1992) *Eur. J. Biochem.* **208,** 699–704
55. Endo, T., Shimada, I., Roise, D., and Inagaki, F. (1989) *J. Biochem.* (Tokyo) **106,** 396–400
56. Dietmeier, K., Hönlinger, A., Bömer, U., Dekker, P. J., Eckerskorn, C., Lottspeich, F., Kübrich, M., and Pfanner, N. (1997) *Nature* **388,** 195–200
57. Dekker, P. J. T., Ryan, M. T., Brix, J., Müller, H., Hönlinger, A., and Pfanner, N. (1998) *Mol. Cell. Biol.* **18,** 6515–6524
58. Hill, K., Model, K., Ryan, M. T., Dietmeier, K., Martin, F., Wagner, R., and Pfanner, N. (1998) *Nature* **395,** 516–521
59. Söllner, T., Pfaller, R., Griffiths, G., Pfanner, N., and Neupert, W. (1990) *Cell* **62,** 107–115
60. Nargang, F. E., Künkele, K.-P., Mayer, A., Ritzel, R. G., Neupert, W., and Lill, R. (1995) *EMBO J.* **14,** 1099–1108
61. Tamm, L. K., and Bartoldus, I. (1990) *FEBS Lett.* **272,** 29–33
62. Roise, D. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 608–612
63. Horwich, A. L., Kalousek, F., Fenton, W. A., Pollock, R. A., and Rosenberg, L. E. (1986) *Cell* **44,** 451–459
64. Roise, D., Horvath, S. J., Tomich, J. M., Richards, J. H., and Schatz, G. (1986) *EMBO J.* **5,** 1327–1334
65. Hammen, P. K., Waltner, M., Hahnemann, B., Heard, T. S., and Weiner, H. (1996) *J. Biol. Chem.* **271,** 21041–21048
66. Káldi, K., Bauer, M. F., Sirrenberg, C., Neupert, W., and Brunner, M. (1998) *EMBO J.* **17,** 1569–1576
67. Davis, A. J., Ryan, K. R., and Jensen, R. E. (1998) *Mol. Biol. Cell* **9,** 2577–2593
68. Schleiff, E., and Turnbull, J. L. (1998) *Biochemistry* **37,** 13052–13058
69. Schleyer, M., and Neupert, W. (1985) *Cell* **43,** 339–350
70. Rassow, J., Hartl, F. U., Guiard, B., Pfanner, N., and Neupert, W. (1990) *FEBS Lett.* **275,** 190–194