Dissection of the Structural Organization of the Aminoacyl-tRNA Synthetase Complex*

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The spatio-temporal organization of proteins within the cytoplasm of eukaryotic cells rests in part on the assembly of stable and transient multiprotein complexes. Here we examined the assembly of the multiaminoacyl-tRNA synthetase complex (MARS) in human cells. This complex contains nine aminoacyl-tRNA synthetases and three auxiliary proteins and is a hallmark of metazoan species. Isolation of the complexes has been performed by tandem affinity purification from human cells in culture. To understand the rules of assembly of this particle, expression of the three nonsynthetase components of MARS, p18, p38, and p43, was blocked by stable small interfering RNA silencing. The lack of these components was not lethal for the cells, but cell growth was slightly reduced. The residual complexes that could form in vivo in the absence of the auxiliary proteins were isolated by tandem affinity purification. From the repertoire of the subcomplexes that could be isolated, a comprehensive map of protein-protein interactions mediating complex assembly is deduced. The data are consistent with a structural role of the three nonsynthetase components of MARS, with p38 connecting two subcomplexes that may form in the absence of p38.

Multiprotein complexes are molecular machines that are essential for organization of the proteome and for integration of cellular functions. Translation of genetic information involves several supramolecular assemblies, including the ribosome and multiprotein complexes involved in the initiation and elongation steps of the protein biosynthesis process (1). A complex containing the four subunits of elongation factor 1 (EF1A and the guanine nucleotide exchange factors EF1βα, EF1ββ, and EF1βγ) and ValRS (valyl-tRNA synthetase) was described (2, 3). Several structural models of the ValRS-EF1A-guanine nucleotide exchange factor assembly have been proposed (2, 4, 5). This complex is believed to play a role in channeling of tRNAVal during translation (6). A multiaminoacyl-tRNA synthetase complex (MARS)4 of about 1.5 MDA was described more than 20 years ago, but its physical and functional organization remain elusive (7). This complex is ubiquitous from Drosophila to mammals and contains the nine aminoacyl-tRNA synthetases ArgRS, AspRS, GluRS, GluΔRS, IleRS, LeuRS, LysRS, MetRS, ProRS, and the three nonsynthetase components p18, p38, and p43 (8). Initial protein-protein interaction maps have been determined by using the yeast two-hybrid system (9, 10) or by in vitro cross-linking (11). A structural working model has been proposed by electron microscopy and three-dimensional reconstruction (12). The p38 component forms the platform for complex assembly (9, 13). A p38 deficiency is lethal in mice (14). Recent studies have indicated that some of the components of this complex lead a double life. They are essential components of translation when associated within MARS but may also play noncanonical functions after dissociation from the complex or following transport in other cellular compartments. LysRS associates with Cu,Zn-superoxide dismutase in familial cases of amyotrophic lateral sclerosis (15) or activates the microphthalmia-associated transcription factor in the nucleus (16). The bifunctional GluProRS polypeptide is recruited within the interferon-γ-activated inhibitor of translation complex during the interferon-γ response (17). The p43 component is released from the complex during apoptosis (18), and is the precursor of the p43(EMAPII) cytokine (19, 20). Parkin, an E3 ubiquitin-protein ligase involved in Parkinson disease, ubiquitylates and promotes the degradation of p38 (21). The finding that components of MARS may oscillate between various functional states led to the suggestion that MARS is a depot for releasable regulatory proteins (22). Thus, the regulation of the spatio-temporal organization of MARS is a key factor for the regulation of the activity of its components. A detailed knowledge of the assembly scheme of this complex is required to understand how association of its components may be regulated by posttranslational modifications.

The deciphering of the structural organization of MARS has been hampered by the high stability of this particle that could...
only be partly dissociated under nondenaturing conditions. Only partial disassembly of the complex was achieved during hydrophobic interaction chromatography (23, 24) or after incubation with chaotropic salts or detergents (25, 26). Some clues concerning the assembly of its components have been obtained by reconstitution of subcomplexes starting from recombinant proteins individually expressed in *Escherichia coli* or in yeast (13).

Affinity purification is a method of choice to isolate macromolecular complexes directly from cell extracts. To further appraise the structural organization of the complexes, these assemblies can be perturbed in vitro after silencing of key components. In order to understand the structural rules that govern in vivo the assembly of the 11 components of MARS, complexes were isolated by combining tandem affinity purification (TAP) with stable transfection of yeast cells expressing 3′/5′-digested with BamHI, HindIII, and digested with BamHI, to create pSG5-CTAP. To create pSG5-NTAP, the TAP tag cassette, recovered from pZome-1-N after digestion with BamHI and Sall and treatment with Klenow, was introduced into pSG5 digested with EcoRI and treated with Klenow. The cDNAs encoding the proteins of interest were introduced into the EcoRI or BamHI sites of pSG5-CTAP or into the EcoRI or BamHI sites of pSG5-NTAP to create fusion proteins carrying a TAP tag appended to the carboxyl-terminal or amino-terminal extremity of the protein, respectively. The cDNAs encoding human p18 (GGGGGATCCATAATGGCGGCGGCCGCAAGAG and GGGGGATCCGTGGGAATTAGTATATAG), p38 (GGGGGATCCATAATGCCGATGTACCAGGTA and GGGGGATCCGTGGGAATTAGTATATAG), p43 (GGGGGATCCATAATGGCAAATAATGATGCTGTT and GGGGGATCCGTGGGAATTAGTATATAG), human p18 (GGGGGATCCATAATGCCGATGTACCAGGTA and GGGGGATCCGTGGGAATTAGTATATAG), and N-p43 (GGGGGATCCGTGGGAATTAGTATATAG) were amplified by PCR with the two oligonucleotides indicated and cloned into pSG5-NTAP. The constructs were verified by DNA sequencing.

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**EXPERIMENTAL PROCEDURES**

*Construction of Stable Cell Lines Expressing TAP-tagged Proteins*—The TAP-tag sequences were introduced into the mammalian expression vector pSG5. The TAP tag cassette of pZome-1-C was excised by EcoRI digestion, Klenow treatment, and BamHI digestion and inserted into pSG5 digested with BglII, treated with Klenow, and digested with BamHI, to create pSG5-CTAP. To create pSG5-NTAP, the TAP tag cassette, recovered from pZome-1-N after digestion with BamHI and Sall and treatment with Klenow, was introduced into pSG5 digested with EcoRI and treated with Klenow. The cDNAs encoding the proteins of interest were introduced into the EcoRI or BamHI sites of pSG5-CTAP or into the EcoRI or BamHI sites of pSG5-NTAP to create fusion proteins carrying a TAP tag appended to the carboxyl-terminal or amino-terminal extremity of the protein, respectively. The cDNAs encoding human p18 (GGGGGATCCATAATGGCGGCGGCCGCAAGAG and GGGGGATCCGTGGGAATTAGTATATAG), p38 (GGGGGATCCATAATGCCGATGTACCAGGTA and GGGGGATCCGTGGGAATTAGTATATAG), and p43 (GGGGGATCCATAATGGCAAATAATGATGCTGTT and GGGGGATCCGTGGGAATTAGTATATAG) were amplified by PCR with the two oligonucleotides indicated and cloned into pSG5-NTAP. The TAP tag cassette, recovered from pZome-1-N after digestion with BamHI and Sall and treatment with Klenow, was introduced into pSG5 digested with EcoRI and treated with Klenow. The cDNAs encoding the proteins of interest were introduced into the EcoRI or BamHI sites of pSG5-CTAP or into the EcoRI or BamHI sites of pSG5-NTAP to create fusion proteins carrying a TAP tag appended to the carboxyl-terminal or amino-terminal extremity of the protein, respectively. The cDNAs encoding human p18 (GGGGGATCCATAATGGCGGCGGCCGCAAGAG and GGGGGATCCGTGGGAATTAGTATATAG), p38 (GGGGGATCCATAATGCCGATGTACCAGGTA and GGGGGATCCGTGGGAATTAGTATATAG), and p43 (GGGGGATCCATAATGGCAAATAATGATGCTGTT and GGGGGATCCGTGGGAATTAGTATATAG) were amplified by PCR with the two oligonucleotides indicated and cloned into pSG5-NTAP. The constructs were verified by DNA sequencing. HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 μg/ml penicillin and streptomycin. Cells were transfected with Effectene (Qiagen). Stable transformants were isolated by selection with G418.

*Isolation of Complexes*—Cells (3 billion) grown in 10 dishes of 140 mm diameter were washed with ice-cold PBS, harvested at 4 °C by mechanical detachment with a cell lifter, washed with ice-cold phosphate-buffered saline, and resuspended in 10 ml of buffer A (10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM MgCl2, 10% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol) containing 0.5% Triton X-100 and protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 2.5 mM benzamidine). Purification of the TAP-tagged proteins was conducted essentially as described previously (27). The lysate was centrifuged at 40,000 × g for 30 min at 4 °C, and 200 μl of IgG-Sepharose 6 Fast Flow (GE Healthcare) were added to the supernatant. After 2 h of incubation at 4 °C, the suspension was applied to a PolyPrep chromatography column (Bio-Rad), and the packed beads were washed five times with 10 ml of buffer A and once with 10 ml of buffer A containing 0.5 mM EDTA. Sepharose beads (200 μl) were incubated overnight at 4 °C, in 200 μl of buffer A containing 0.5 mM EDTA and the TEV protease at a final concentration of 100 μg/ml. The eluate (200 μl) was recovered, and beads were washed with 200 μl of buffer A containing 0.5 mM EDTA. The combined eluates (400 μl) were diluted by the addition of 3 volumes of buffer B (10 mM Tris-HCl, pH 8.0, 1 mM imidazole-HCl, pH 8.0, 50 mM NaCl, 5 mM MgCl2, 1 mM magnesium acetate, 2 mM CaCl2, 10% glycerol, 0.1% Nonidet P-40, 10 mM 2-mercaptoethanol) and incubated for 2 h at 4 °C after the addition of 200 μl of calmodulin-Sepharose 4B (GE Healthcare) and of CaCl2 to a final concentration of 2 mM. Beads were then washed five times with 10 ml of buffer B, and protein complexes were eluted after a 5-min incubation in 200 μl of buffer C (10 mM Tris-HCl, pH 8.0, 1 mM imidazole-HCl, pH 8.0, 50 mM NaCl, 5 mM MgCl2, 1 mM magnesium acetate, 0.1% Nonidet P-40, 10 mM 2-mercaptoethanol, and 3 mM EGTA). Elution was repeated five times, the various eluates were pooled, and proteins were precipitated by the addition of 0.25 volumes of a solution containing 50% (v/v) trichloroacetic acid and 0.05% (v/v) Triton X-100. After incubation on ice for 30 min, the precipitate was recovered by centrifugation, washed four times with 500 μl of diethyl ether, air-dried, and resuspended in the loading buffer for analysis by SDS-PAGE. Proteins were separated by SDS-PAGE and visualized by staining with Coomassie Blue R250.

*Identification of Polypeptides by Mass Spectrometry*—In-gel digestion and MALDI-TOF mass spectrometry were performed essentially as described (28). Briefly, bands were excised and digested by several incubations in 100 μM ammonium bicarbonate, pH 8.5, containing 50% acetonitrile. Then proteins were reduced with dithiothreitol, and cysteines were carbamidomethylated. Tryptic digestion was performed overnight at 37 °C in 50 mM NH4HCO3 with 150 ng of trypsin. The supernatants were removed, and peptides were extracted with 1% trifluoroacetic acid and then with 60% acetonitrile containing 1% (v/v) trifluoroacetic acid. The combined extracts were then reduced to ~5–10 μl under vacuum. 1.5 μl of tryptic samples was mixed with 1.5 μl of saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.3% trifluoroacetic acid, and 1.5 μl of this premix were then deposited onto the
sample plate and allowed to dry at room temperature. Spectra were acquired on a Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems) equipped with a 337-nm nitrogen laser, in positive ion reflector mode with delayed extraction. External calibration was applied, based on a mixture of six reference peptides covering the m/z 900–3700 Da range. Proteins were identified using the search program Mascot (available on the World Wide Web), reducing the Swiss-Prot/TrEMBL and NCBI data bases to the Homo sapiens species. Two missed cleavage sites, possible oxidation of methionine, and modification of cysteines by iodoacetamide were considered in searches.

Construction of Knockdown Strains for the p18, p38, and p43 Components of MARS—Oligonucleotides encoding human siRNA for p18 (GATCCCCACAATGGTCCAAGTCGTTCCAAAAATAAGAGTTAGACTTGGACCATTTTGTGAAC and TCGAGTTCCAAAAAACAATGGTCCAAGGG), p43 (GATCCCCGAATGTGCCGAAGACGCAGTTCTAACCTCAAGACGTGCTTCGGCACATTCGGG), and p38 (GATCCCCGAATGTGCCGAAGACGCAGTTCTAACCTCAAGACGTGCTTCGGCACATTCGGG) were annealed and adsorbed on a calmodulin column in the presence of Ca2+ ions immobilized IgGs, released by TEV protease digestion, starting from 2–3 billion cells. Fusion protein was captured on the complex (34). Tandem affinity purification was conducted using other TAP-tagged components also exposed on the outside of the particle (9, 32, 33). Concerning p38, the scaffold component of MARS (9), two fusions were generated, with insertion of the TAP tag either at the N or C terminus of the protein. Each individually tagged protein was expressed in HeLa cells after stable integration of the DNA constructs. To avoid overexpression of the TAP-tagged protein as compared with its protein partners, HeLa strains expressing the fusion proteins at nearly physiological levels as compared with the endogenous proteins were selected (result not shown). This resulted in the isolation of homogeneous populations of complexes (see below).

First, we purified MARS using the MetRS-CTAP fusion protein, a component known to be present as a single copy within the complex (34). Tandem affinity purification was conducted starting from 2–3 billion cells. Fusion protein was captured on immobilized IgGs, released by TEV protease digestion, adsorbed on a calmodulin column in the presence of Ca2+, and eluted by the addition of EGTA. The polypeptides copurified with MetRS-CTAP were identified by mass spectrometry following in-gel digestion with trypsin. The polypeptide composition of the complex isolated from human cells with MetRS-CTAP was very similar to the composition of the rabbit complex purified according to standard procedures (Fig. 1).

The finding that similar MARS complexes, always containing the same 11 major polypeptides, were obtained when purification was conducted using other TAP-tagged components also argues that MARS is a structurally well-defined particle. The complexes obtained using p18-CTAP or p43-CTAP contained the same polypeptides as previously observed with MetRS-CTAP (Fig. 1). Because p18 and p43 are dimers and the fusion proteins were expressed to close-to-endogenous levels, the purified complexes contained a mixture of the native endogenous protein and of the recombinant fusion proteins containing the calmodulin-binding peptide (white and black arrowheads in Fig. 1, respectively). We suggested previously that the p38 protein is the scaffold for the assembly of MARS (9, 13). One of the possible models for the association of the 10 other components to p38 considered that p38 constitutes the core of this assembly that could form a compact structure around its scaffold protein. We were concerned about the possibility that introduction of a TAP tag, an appended domain of 20 kDa, to the N or C terminus of p38 might destabilize the association of some of the components of the complex. Thus, we performed the purification of complexes using p38-CTAP or p38-NTAP (Fig. 1). The complexes isolated with these two constructs contained the same 11 polypeptide components. The yield of complex recovery was lower with p38-NTAP, as compared with p38-CTAP (Fig. 1), suggesting a limited accessibility within the complex of the TAP
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The more potent inhibition was selected for further studies. Then the construct leading to ability to stably and specifically inhibit the expression of the 21-nucleotide-long target sequences/gene were tested for their grate into the genome (29). Six constructs carrying different over time, we expressed siRNA with retroviral vectors that inte-

To create mutant cell lines in which silencing would be stable 

doubling time of 18 h at 37 °C, HeLa/p18si-, HeLa/p38si-, and 

MARS (rabbit) MetRS-CTAP p18-CTAP p43-CTAP p38-CTAP p38-NTAP

FIGURE 1. Purification of cellular MARS complexes. SDS-PAGE analysis of MARS purified from HeLa cells expressing MetRS-CTAP, p18-CTAP, p43-CTAP, p38-CTAP, or p38-NTAP. The black arrowheads denote the pro-
teins of MARS used for the purification by the TAP tag procedure. They contain the calmodulin-binding peptide remaining after TEV protease cleavage. The white arrowheads point to the endogenous proteins that do not contain a tag. Proteins were stained with Coomassie Blue. The MARS complex purified from rabbit liver by a conventional procedure (32) and identity of its components (8) are indicated on the left.

tag appended to the N terminus of p38 for binding to the affinity columns. The finding that no additional polypeptide is copurified in stoichiometric amounts with the various TAP-tagged proteins used in this study clearly shows that MetRS, p18, p38, and p43 are associated within the same particle in the cellular environ-

mental, in the physiological context of exponential cell growth.

Translational Silencing of the Auxiliary Components of MARS—To investigate the consequence of knockdown expres-
sion of the auxiliary components of MARS on the viability of human cells in culture and on the assembly of MARS in cellulo, we searched for siRNA capable of inhibiting the expression of p18, p38, or p43 to a steady-state level that could not be detected by Western blotting. The MARS complex displays a high stability in cellulo, with a cellular half-life of about 48 h corresponding to three cell division times (results not shown). To create mutant cell lines in which silencing would be stable over time, we expressed siRNA with retroviral vectors that inte-

rate into the genome (29). Six constructs carrying different 

21-nucleotide-long target sequences/gene were tested for their ability to stably and specifically inhibit the expression of the corresponding gene products. Then the construct leading to the more potent inhibition was selected for further studies.

First, we noticed that silencing of p18 (p18si), p38 (p38si), or p43 (p43si) in HeLa cells to a cellular level that could not be detected with antibodies directed to these components of MARS (Fig. 2) was not lethal for the cell. No strong phenotypic effect was observed. Only a growth retardation phenotype could be observed. Whereas wild-type HeLa cells displayed a doubling time of 18 h at 37 °C, HeLa/p18si, HeLa/p38si, and HeLa/p43si-stable cell lines showed doubling times of 24, 24, and 23 h, respectively, correspon-
ding to a 30% decrease in growth rate.

The steady-state level of the components of MARS in HeLa cells sub-

ected to p18, p38, or p43 silencing was compared with normal cells (Fig. 2). The first noticeable feature is that the knockdown of one of the auxiliary components of the complex did not dramatically disturb the steady-state level of the other components. The most discernible side effects were observed on the steady-state level of p43 and AspRS in HeLa/p38si cells (Fig. 2). This is most certainly related to the weaker cellular stability of these compo-

nents when, in the absence of the scaffold protein p38, they are no more associated within MARS (see below).

Mapping the Assembly of MARS in Vivo—Each siRNA was expressed in the HeLa cell lines described above that had stably integrated the DNA constructs that express the fusion protein MetRS-CTAP, p43-

CTAP, p38-CTAP, or p18-CTAP, and stable cell lines were selected. The in vivo assembly of the MARS complex in the absence of its p18, p38, or p43 component was explored by isolating the various TAP-tagged components in combination with the expression of the three siRNAs. In total, nine distinct TAP purifications were performed and at least two purifica-
tions per combination of TAP tag and siRNA species.

To explore the putative structural role of p18 in the assembly of MARS, we isolated subcomplexes with MetRS-CTAP, p43-

CTAP, or p38-CTAP (Fig. 3A) in a p18si background. MetRS was the only polypeptide recovered from HeLa/MetRS-CTAP/
p18si (Fig. 3A), and MetRS and p18 were the only polypeptides 

that were absent in the complexes recovered from HeLa/p43-

CTAP/p18si or HeLa/p38-CTAP/p18si (Fig. 3A). Thus, p18 serves to anchor MetRS to the other components of MARS. When p18-CTAP or MetRS-CTAP was used to purify complexes in a p43si background, the same subcomplexes containing GluProRS, IleRS, LeuRS, LysRS, AspRS, and p38, in addition to p18 and MetRS, were isolated (Fig. 3A). In a p38si back-
ground, only GluProRS, IleRS, and LeuRS were recovered in a complex containing p18 and MetRS (Fig. 3A), showing that LysRS and AspRS require p38 to associate with these compo-

nents. Conversely, in this p38si background, another subcom-
plex was isolated with p43-CTAP, containing p43, GlnRS, and 

ArgRS (Fig. 3A).

On the basis of this network exploration, the following scheme of MARS assembly can be proposed (Fig. 3B). Two subcomplexes could be isolated in the absence of the scaffold protein p38 and thus are likely to be able to assemble in vivo in the absence of p38; subcomplex I contains MetRS, p18, GluProRS, IleRS, and LeuRS, and subcomplex II is composed of p43,
GlnRS, and ArgRS. Association of either LysRS or AspRS to these complexes requires p38. In line with this observation, the possibility of forming stable subcomplexes containing only p38 and LysRS or containing p38 and AspRS has been previously described (13). Thus, p38 has an essential scaffolding function, not only in vitro (9) but also in vivo. The auxiliary protein p18 also fulfills an important structural role. It is involved in anchoring MetRS to the other components of MARS. Concerning p43, additional TAP tag fusion proteins were constructed to understand its structure/function relationships.

**Mapping the Domain Structure of p43**—The p43 subunit of MARS is a 312-amino acid protein with tRNA-binding properties (32, 35). This polypeptide is cleaved after Ser106 during apoptosis (18). The C-terminal domain, p43(ARF), is released from the cells and is converted into p43(EMAPII), a putative cytokine (19). Whether the truncation of p43 is accompanied by the total or partial fusion of the MARS complex or not and whether the truncated p43(ARF) domain associates with other cellular components before its release from the cell or not remained to be established.

Np43-CTAP corresponds to the N-terminal domain of p43, from residue 1 to 106. When expressed in HeLa cells with a TAP tag merged at its C terminus, a position naturally occupied by p43(ARF), the C-terminal domain of p43. The complex isolated using Np43-CTAP was identical to that described above, isolated with p43-CTAP, the full-length protein (Fig. 4). Thus, formation of subcomplex II, containing p43, GlnRS, and ArgRS, and its association with the other components of MARS is likely to only involve the N-terminal moiety of p43. Accordingly, when the C-terminal domain of p43, starting at residue 107, was expressed in HeLa cells with the TAP tag inserted at the C terminus, no polypeptide was copurified with p43(ARF)-CTAP (Fig. 4). This result clearly showed that the C-terminal domain of p43 does not have the capacity to associate with components of MARS after cleavage from the N-domain and that p43(ARF) does not possess the ability to form alternative complexes after its release from MARS during apoptosis. Thus, the N-terminal domain of p43 is an important building block of MARS that serves to anchor GlnRS and ArgRS to the complex. In addition, linking Np43 to p43(ARF) also results in the recruitment of a tRNA-binding cofactor to the complex.

**DISCUSSION**

We have performed an *in vivo* analysis of the assembly of MARS. The complexes isolated with TAP-tagged MetRS, p18, p38, or p43, are similar. This result suggests that the three non-synthetase components of MARS do not participate in other major multiprotein assemblies in exponentially growing cells. Our results are also consistent with a structural role of the three nonsynthetase components of MARS. The p18 subunit anchors MetRS to the complex, p43 brings GlnRS and ArgRS to the complex, and p38 connects the two subcomplexes I (MetRS, p18, GluProRS, IleRS, and LeuRS) and II (p43, GlnRS, and ArgRS) that can form *in vivo*, with AspRS and LysRS (Fig. 3B). Our previous mapping of the protein binding sites on p38 (9) suggests that the C-terminal moiety of p38 associates with subcomplex I, whereas its N-terminal domain interacts with subcomplex II and with LysRS and AspRS. Because these complexes were isolated by TAP performed at low ionic strength, our results strongly suggest that MARS is a stable and discrete macromolecular entity that does not arise from a larger complex comprising the 20 aminocyl-tRNA synthetases.

Initial models of MARS assembly were proposed on the basis of two-hybrid (9, 10) or cross-linking experiments (11). In the latter study, a three domain model was proposed. Domain I contained GluProRS, LeuRS, and IleRS; domain II was made of MetRS, GlnRS, and AspRS; and domain III associated ArgRS and LysRS. Domain I corresponds to subcomplex I lacking MetRS and p18 (p18 was not taken into account into that study), but subcomplex II does not correspond to either domain II or domain III. The discrepancies between these two sets of results is certainly due to the methods used to identify the protein subcomplexes. The cross-linking method requires the presence of chemical groups that should be reactive and located at a distance compatible with the length of the cross-linking agent but does not require a direct interaction between the two components. The protein subcomplexes are then deduced from the set of pairwise cross-links that were identified. The
approach of copurification that we used in this study is a more direct one to identify subcomplexes but does not directly address the possibility of direct interaction. By contrast, pairwise interaction is exactly what is observed by the two-hybrid system. Using this approach, we previously reported that ArgRS and GlnRS do interact with p38 (Fig. 3B) (9), but the dissociation constants determined by Biacore were very high (\(>5 \mu M\)) as compared with those determined with p43 (about 100 nM) (13). The weakness of these associations could explain the fact that these two components were not recovered in the complexes isolated with p18-CTAP or MetRS-CTAP in a p43\(^{si}\) background (Fig. 3A) and exemplifies the synergistic contribution of multiple interactions in the stabilization of the particle (13). Similarly, the finding that AspRS is not recovered in the subcomplexes isolated in a p38\(^{si}\) background (Fig. 3A), whereas a direct interaction with GluProRS was observed by two-hybrid experiments (Fig. 3B) (9), also stresses the essential role of p38 as a scaffolding protein. Thus, combining different approaches is essential to obtain complementary information in order to decipher the structural organization of a multienzyme complex.

The results reported in this work, which provide some clues as to the assembly process of MARS in vivo, are of general interest. These experiments demonstrate that the architecture and function of multienzyme complexes can be probed in vivo by combining TAP and siRNA approaches. We also showed that a more detailed structural organization of one of the components of a complex can be efficiently investigated by this method. The loss of p43, a procytokine, resulted in partial dissociation of MARS, but the N-terminal domain of p43 was sufficient to restore the integrity of the complex. Thus, the portion of p43 released during the course of apoptosis, after cleavage of p43 after Ser\(^{106}\) (18), is not crucial for complex formation. The finding that p43(ARF) does not form alternative complexes after its release from MARS is consistent with the finding that it does not accumulate in cells and is readily recovered in the supernatant of apoptotic cells (18).

This first in vivo physical map of MARS was obtained by combining TAP with the silencing of p18, p38, and p43 with siRNA. Silencing of these components to a level undetectable by Western blotting shows that these components are not essential to the viability of cultured cells but also demonstrates that the in vivo stability of the other subunits of MARS is not drastically affected by partial or complete disruption of the complex. This conclusion is in contrast with previous analyses suggesting that the integrity of the MARS particle is an essential...
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FIGURE 4. Dissecting domain structure of p43 required for MARS assembly. The C-terminal (p43(ARF)-CTAP) or N-terminal (Np43-CTAP) domains of p43 were stably expressed in HeLa cells and isolated by the TAP tag procedure. The black arrowheads denote the p43 derivatives used for TAP. The control purification with p43-CTAP, shown in Fig. 1, is reproduced on the right.

factor of the stability of its components (36). In this earlier work, HeLa cells have been transfected with siRNAs directed to nonsynthetase as well as to synthetase components of MARS, and the stability of its polypeptide components has been analyzed on the total population of cells, including transfected and nontransfected cells, 48 h after transfection. In that case, it is not known whether the decrease of the steady-state level of one component is the consequence of its release from the complex or is the consequence of the onset of necrosis, as expected when expression of essential enzymes, such as aminoacyl-RNA synthetase as well as to synthetase components of MARS, is the consequence of its release from the complex. These results suggest that in multienzyme organisms, the assembly of MARS may regulate the activity of its components and the balance between their canonical and noncanonical functions. When MARS is constitutively disrupted after inactivation of one of its structurally important building blocks, the availability in a nonregulated manner of free, individual components displaying a potential deadly double life could be deleterious at some stage of the development of multienzyme organisms.

it is worth noting that depletion of p38 is lethal, but fibroblasts obtained from 13.5-day embryos are viable (14). As indicated in the Introduction, several components of MARS are involved in regulatory pathways when dissociated from the complex. These results suggest that in multienzyme organisms, the assembly of MARS may regulate the activity of its components and the balance between their canonical and noncanonical functions. When MARS is constitutively disrupted after inactivation of one of its structurally important building blocks, the availability in a nonregulated manner of free, individual components displaying a potential deadly double life could be deleterious at some stage of the development of multienzyme organisms.

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