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Hierarchical Network between the Components of the Multi-tRNA Synthetase Complex

IMPLICATIONS FOR COMPLEX FORMATION[*S]

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Jung Min Han[‡1], **Min Ji Lee**[§1], **Sang Gyu Park**[§], **Sun Hee Lee**[§], **Ehud Razin**[¶], **Eung-Chil Choi**[§], **and Sunghoon Kim**[§2]

From the [‡]*Imagene Company Biotechnology Incubating Center, Golden Helix, Seoul National University, Seoul 151-741, Korea, the* [§]*National Creative Research Initiatives Center for ARS Network, College of Pharmacy, Seoul National University, Seoul 151-741, Korea, and the* [¶]*Department of Biochemistry, Hebrew University Hadassah Medical School, Jerusalem 91120, Israel*

The macromolecular tRNA synthetase complex consists of nine different enzymes and three non-enzymatic factors. This complex was recently shown to be a novel signalosome, since many of its components are involved in signaling pathways in addition to their catalytic roles in protein synthesis. The structural organization and dynamic relationships of the components of the complex are not well understood. Here we performed a systematic depletion analysis to determine the effects of structural intimacy and the turnover of the components. The results showed that the stability of some components depended on their neighbors. Lysyl-tRNA synthetase was most independent of other components for its stability whereas it was most required for the stability of other components. Arginyl- and methionyl-tRNA synthetases had the opposite characteristics. Thus, the systematic depletion of the components revealed the functional reason for the complex formation and the assembly pattern of these multi-functional enzymes and their associated factors.

Since many cellular proteins exert their biological effects via the formation of macromolecular complexes, it is important to understand the pattern of assembly and dynamic relationships of the components of such complexes. Aminoacyl-tRNA synthetases (ARSs)[3] are essential enzymes catalyzing the ligation of their cognate amino acids and tRNAs in the process of translation. In higher eukaryotes, nine different enzymes form a macromolecular complex with three non-enzymatic factors (ARS-interacting multifunctional protein): AIMP1/p43, AIMP2/p38, and AIMP3/p18 (1, 2).

Although the existence of this complex has been known for many years, the reason for its formation and the connections between its components remain unclear. Many of the enzymes of the complex perform other roles in addition to their enzymatic functions. For instance, EPRS (glutaminyl-prolyl-tRNA synthetase) forms a novel gene silencing complex with ribosomal subunit L13a and GAPDH (3) when the cells are exposed to IFN-γ (interferon-γ). QRS (glutaminyl-tRNA synthetase) and MRS (methionyl-tRNA synthetase) are involved in anti-apoptotic regulation and rRNA biogenesis, respectively (4, 5). KRS (lysyl-tRNA synthetase) has the most diverse activities of the complex-forming enzymes, activating mast cells by generating Ap4A as a secondary catalytic product (6), being involved in human immunodeficiency virus assembly via interaction with the viral Gag protein (7), and being secreted as a pro-inflammatory cytokine (8). In addition, the ARS-interacting factors AIMP1, -2, and -3 occupy individual niches in cell regulation. For instance, AIMP1 acts as a cytokine with diverse activities (9–11) and as a systemic hormone involved in glucose homeostasis (12). AIMP2 down-regulates c-Myc during lung cell differentiation (13) and is a target substrate for Parkin in the control of neuronal cell death (14), while AIMP3 is a tumor suppressor that activates ATM/ATR, which is required for repair of damaged DNA (15, 16).

In view of the multifunctional nature of these essential enzymes and factors, understanding their assembly should provide important insight into the mechanism of protein synthesis and into cellular regulatory mechanisms. Several approaches have been used to probe the assembly of the complex (17–24). One proposed model of its structural organization derived from these studies divides the complex into three subdomains (19). DRS, MRS, and QRS are placed in one subdomain, the second is composed of KRS and RRS, and the third of the high molecular weight EPRS, IRS, and LRS. The three AIMPs, AIMP1/p43, AIMP2/p38, and AIMP3/p18, are thought to connect the subdomains and stabilize the overall structure. AIMP1 is located in the middle of the complex (24) and associates with RRS (arginyl-tRNA synthetase) via its N-terminal region (25). AIMP2 is linked to many components (17, 20) and was shown to be critical for the assembly of the complex (26). The contribution of AIMP3 to complex formation is yet to be determined.

To understand the structural significance of each component for complex formation and the reason for its formation, we systematically depleted each component by the siRNA tech-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Methods and supplemental Figs. 1–4 and Table 1.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed. Tel.: 82-2-880-8180; Fax: 82-2-875-2621; E-mail: sungkim@snu.ac.kr.

[3] The abbreviations used are: ARS, aminoacyl-tRNA synthetase; AIMP, ARS-interacting multi-functional protein; siRNA, small interfering RNA; RNAi, RNA interference; EPRS, glutaminyl-prolyl-tRNA synthetase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; QRS, glutaminyl-tRNA synthetase; MRS, methionyl-tRNA synthetase; KRS, lysyl-tRNA synthetase; DRS, aspartyl-tRNA synthetase; RRS, arginyl-tRNA synthetase; IRS, isoleucyl-tRNA synthetase; LRS, leucyl-tRNA synthetase; WRS, tryptophanyl-tRNA synthetase; RT, reverse transcription.

Supplemental Material can be found at: http://www.jbc.org/cgi/content/full/M605211200/DC1

nique and determined how this affected the stability of the other components, as well as protein synthesis. The results revealed that the stability of the components is affected by formation of the complex but that the extent of the dependence varies among the components. KRS was found to be the most independent of complex formation for its stability and at the same time the most important for the stability of the complex as a whole, whereas RRS and MRS had the opposite characteristics, suggesting some form of hierarchical arrangement of the complex.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa cells derived from cervical cancer cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 μg/ml penicillin and streptomycin in a 5% CO_2 incubator.

Western Blot Analysis—For Western blotting, HeLa cell lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 10% glycerol, 10 μM sodium fluoride, 10 μM β-glycerophosphate, 1 μM sodium orthovanadate (Na_3VO_4), and protease inhibitors). Whole cell lysates were boiled in 5× sample buffer (50 mM Tris-HCl, pH 6.8, 86 mM β-mercaptoethanol, 10% glycerol, and 2% SDS) for 5 min. 40 μg of protein extract per lane was subjected to electrophoresis, transferred to a polyvinylidene fluoride membrane (Millipore), and immunoblotted with antibodies specific to AIMP1, AIMP2, AIMP3, KRS, RRS, QRS, MRS, LRS, IRS, and EPRS. As a control, the same membrane was stripped and immunoblotted with anti-tubulin antibody (Santa Cruz Biotechnology). The membrane was washed and treated with horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies (Pharmacia Corp.).

RNA Interference (RNAi)—RNAi duplexes corresponding to AIMP1, AIMP2, AIMP3, KRS, RRS, QRS, MRS, LRS, IRS, and EPRS were synthesized (Invitrogen). For the siRNA experiment, 2×10^5 HeLa cells were plated in 6-well plates (dishes), and all transfections of siRNA (20 pM) were performed using Lipofectamine 2000 (Invitrogen). Cells were harvested after 48 h. The effect of siRNA on the expression of ARS complex components was measured by Western blot analysis. As a control, we used Stealth RNAi Negative Control Duplexes (Invitrogen).

Quantitative RT-PCR—The transcript levels of the components of the multi-tRNA synthetase complex were determined by quantitative RT-PCR. Total RNAs were isolated from siRNA-transfected cells and converted to cDNAs using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and an anchored oligo(dT) primer set. The resulting cDNAs were used as templates for PCR ampification with specific primer pairs. As a control, GAPDH was also quantified by RT-PCR from the same RNA samples.

Gel Filtration Chromatography—The proteins extracted from cells were subjected to sizing chromatography using Sephacryl S-300 (separation range 10–1,500 kDa) in an AKTA FPLC system (Pharmacia Corp.), as described previously (8). The separated proteins were resolved by SDS-PAGE and subjected to Western blotting with antibodies specific for each of the components.

TABLE 1
siRNA sequences targeting each of the components of the multi-tRNA synthetase complex

Target	siRNA sequence (5′ to 3′)
AIMP3	CCA AGU CUA ACA GGA UUG ACU ACU A
AIMP2	ACA CCA GAU GCA GAC UUG GAU GUA A
AIMP1	GGA GCU GAA UCC UAA GAA GAA GAU U
KRS	GCU GUU UGU CAU GAA GAA AGA GAU A
RRS	GGC UGU UUA GGA GCU UCU CCA AAU U
QRS	GCC AUC AAU UUC AAC UUU GGC UAU G
MRS	CUA CCG CUG GUU UAA CAU UUC GUU U
LRS	CCU UGC AUG GAU CAU GAU AGA CAA A
IRS	GGA AGC CAG AUU GUC AGC CCU CUA U
EPRS	CCA GCA CUA CCA GGU UAA CUU UAA A

RESULTS AND DISCUSSION

Systematic Depletion of Components of the Multi-ARS Complex—We depleted each component of the complex with siRNA. To determine the most effective siRNA for suppressing each of the components, we prepared three different siRNAs designed to target each transcript, tested their efficacy (data not shown), and selected the most effective siRNA (Table 1). Since four different siRNAs targeting the DRS transcript did not have sufficient suppressing effects, for unknown reasons (data not shown), we excluded DRS from the analysis. Apart from the siRNA that only suppressed the QRS transcript to about 55% of the control, all the other siRNAs silenced their target mRNAs adequately in HeLa cells, as demonstrated by quantitative RT-PCR (Fig. 1*A*) and real-ime RT-PCR (supplemental Table 1 and supplemental Fig. 1). Suppression of each transcript did not reduce expression of the other components (Fig. 1*A* and supplemental Fig. 1).

The Effect of Depletion of AIMPs—AIMP2 has been shown by *in vivo* analysis (26) to be critical for the integrity of the whole complex and the stability of its components. It interacts with AIMP1 via a coiled-coil interaction (27). We found that depletion of any one of the three AIMPs greatly affected the levels of the others (Fig. 1*B*, supplemental Fig. 2, and Fig. 2, *A* and *B*) and also had a global effect on the stability of the enzymatic components apart from KRS, although the degree of disruption differed for the three factors (Fig. 1*B*, supplemental Fig. 2, and Fig. 2*A*). These results show that the three AIMPs are critical for the assembly and stability of the whole complex.

The Effect of Depletion of ARSs—EPRS, IRS, and LRS are larger than the other complex-forming enzymes and are thought to form one subdomain. These three enzymes were also shown to be mutually dependent for their stability (Fig. 1*B*, supplemental Fig. 2, and Fig. 2*A*). IRS was most sensitive to depletion of the other two enzymes. These results could be due to differences in intrinsic stability. However, assuming that these three enzymes have similar intrinsic stabilities, this suggests that IRS is a linker connecting EPRS and LRS (Fig. 2*C*). The three AIMPs were also severely affected by depletion of these large enzymes, suggesting that the latter are also linked directly or indirectly to the AIMPs.

Of the rest of the component enzymes, MRS and RRS appeared to be least necessary for the stability of the remaining components, but they themselves depended on the presence of the other components. However, RRS was required to stabilize AIMP1, its specific interacting partner (28), suggesting a functional explanation for their association (Figs. 1*B* and 2*A*). In

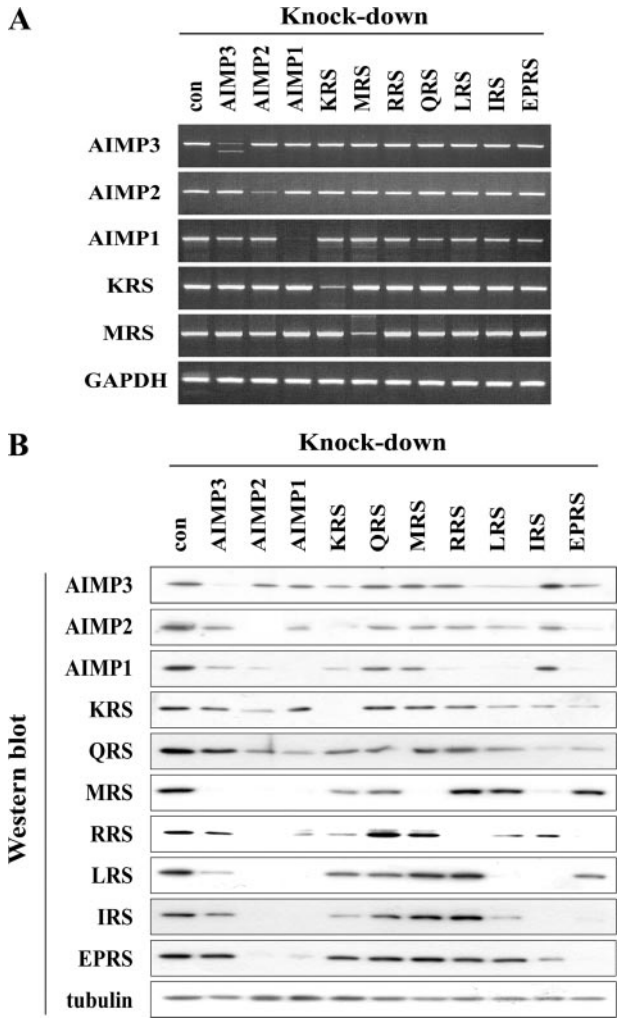

FIGURE 1. **The effect of siRNAs targeting the components of the multi-ARS complex.** *A*, the effects of each siRNA on the transcript levels of the components were assessed by quantitative RT-PCR. Total RNAs were isolated from the siRNA-transfected HeLa cells, converted to cDNAs, and PCR amplified with their specific primers. GAPDH was used as a control. The data shown are representative of three separate experiments. *B*, 2×10^5 HeLa cells were transfected with each siRNA (20 pM). After 48 h, the effect on the levels of the components was determined by Western blot analysis. The amount of protein extract used was in the linear range for immunoblot analysis with all of the antibodies used. *White boxes* indicate the effect of the siRNA on expression of the target. The data shown are representative of three separate experiments.

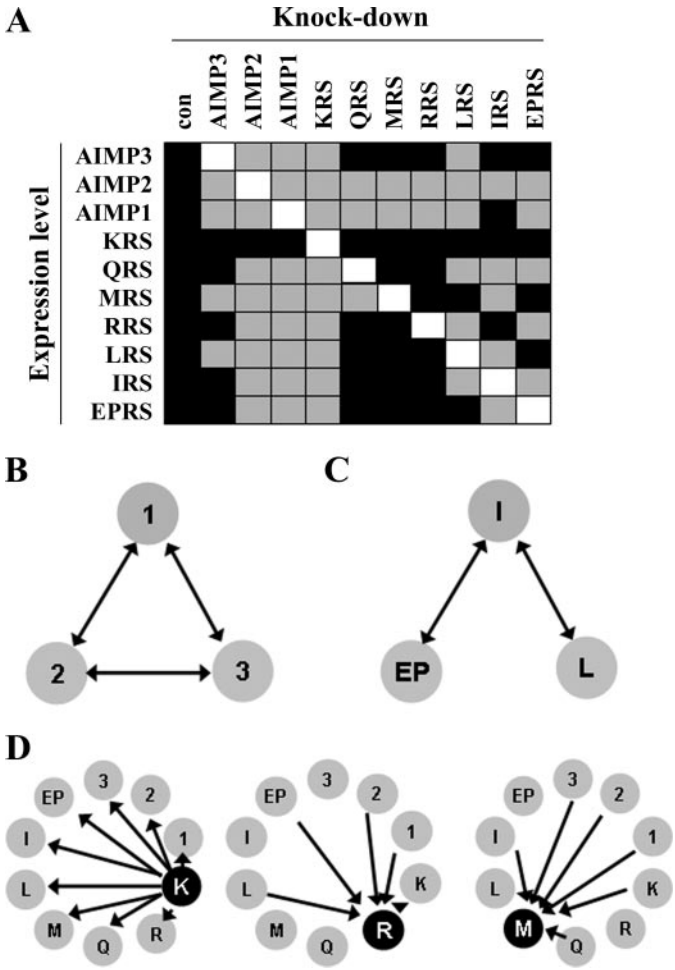

FIGURE 2. **Schematic representations of the effect of depletion on the levels of components.** *A*, *white boxes* indicate the knockdown targets. *Black* and *gray boxes* indicate those not affected and significantly (over 40% of the control) suppressed by the knock-down of the target components, respectively. *B*, the stability relationship between the three non-enzymatic factors, AIMP1, -2, and -3. These factors are mutually dependent for their cellular stability. *C*, the interdependence of the three large enzymes, EPRS, IRS, and LRS, is shown. Of these three enzymes, IRS appears to be positioned at the center, linking the two others. EPRS and LRS did not show mutual dependence. *D*, KRS is not dependent on other components for its stability, whereas it is required for the stability of the other components except for EPRS. In contrast, RRS and MRS are not required for the stability of the other components but are heavily dependent on other components for their stability.

contrast KRS was not affected by depletion of any other component but was required for the stability of the other components (Fig. 1*B*, supplemental Fig. 2, and Fig. 2*A*). Based on these results, the components of the multi-ARS complex appear to be associated in a hierarchical manner with KRS occupying the controlling position and enzymes such as RRS and MRS at the most dependent level (Fig. 2*D*). WRS, which is not a component of the complex, was not affected by the presence or absence of the complex-forming ARSs (data not shown), suggesting that the complex-forming characteristics of the ARSs are related to their intrinsic instability.

The Effect of Component Depletion on Complex Formation—To determine how knock-down of its components affects complex formation, we assessed complex formation by sizing chromatography. Components bound to the intact ARS complex elute in the void volume of the gel filtration column, whereas dissociated components appear in later fractions, depending on their molecular weights. Since the three AIMPs are thought to be important for the integrity of the complex, we investigated how suppression of each AIMP affected complex formation. To avoid protein degradation, the cells were treated with the proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucinal. All the components of the complex from control siRNA-transfected cells co-eluted in the void volume (Fig. 3). However, transfection of siRNAs specific for each AIMP resulted in the dissociation of several components, especially MRS, QRS, KRS, and RRS (Fig. 3), further suggesting the importance of these factors for the assembly of the complex.

The Effect of Component Depletion on Protein Synthesis—To determine how knock-down of the components affected protein synthesis, we compared levels of [^{35}S]methionine incorpo-

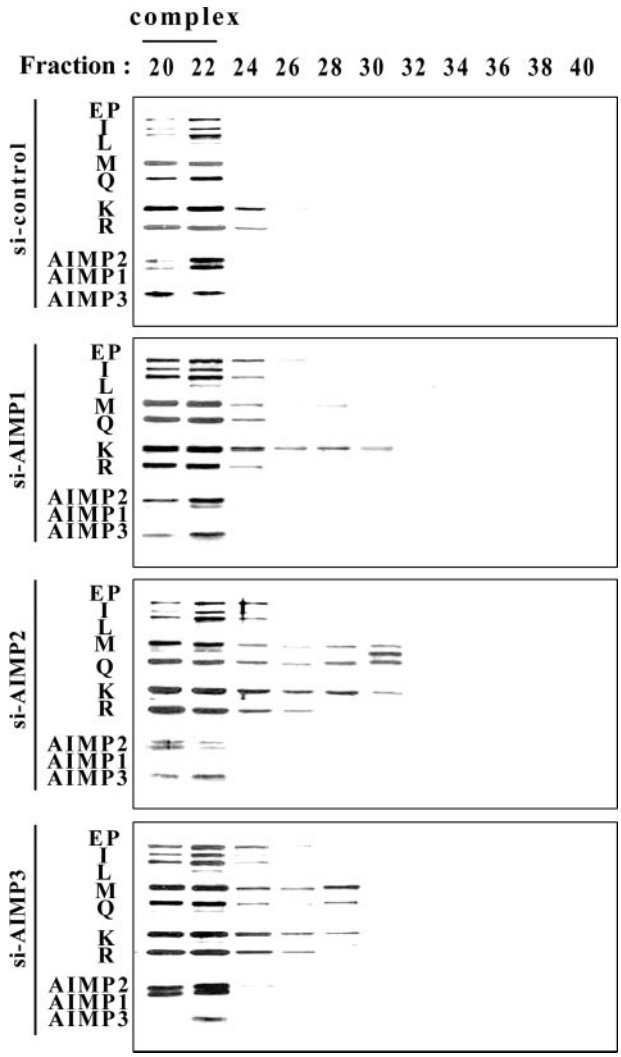

FIGURE 3. **The effect of siRNAs targeting the three AIMPs on complex formation by the ARSs.** 6×10^6 HeLa cells were transfected with the indicated siRNAs. After 18 h, cells were treated with the proteasome inhibitor *N*-acetyl-leucyl-leucyl-norleucinal for 6 h, and the effects of the siRNAa on complex formation were determined by gel filtration. The proteins eluted in each fraction were separated by SDS-PAGE, and each component was detected by Western blotting with specific antibody. The data shown are representative of three separate experiments.

ration in control and ARS siRNA-transfected cells. Although knock-down of each component suppressed protein synthesis to different degrees in the range of 30–80% of the control cells, it did not completely block protein synthesis, even though each component was suppressed to levels almost undetectable by Western blotting (except for QRS) (supplemental Fig. 3). This result indicates that protein synthesis is quite resistant to variation of ARS levels.

Linkage Patterns and Functional Implications for ARS Assembly—The results of the component depletion experiments suggest that the components of the multi-tRNA synthetase complex are heterogeneous in their linkage patterns and intrinsic cellular stability. Although depletion of each component affected the levels of other components to different degree depending on the cell type and growth condition, the depletion effects were consistent in different experiments (data not shown) and reflect the physical intimacy of the components. Among the components, AIMP1 and AIMP3 in addition to AIMP2 (26) were found to be critical for the stability of the whole complex. However, their stability also depended on the presence of other enzymes in the complex. KRS differed from all the other components as it did not require the other components for its stability but was instead essential for formation of the complex. In contrast, the enzymes like RRS and MRS appear to be strongly dependent on complex formation for their stability. The middle-sized components such as MRS, QRS, KRS, and RRS appeared to be easily released from the complex. Interestingly, the dissociated forms of these enzymes have also been detected in physiological conditions (4, 5, 8, 29). Thus each component seems to be linked to the complex in a unique manner (supplemental Fig. 4). Perhaps, this unique linkage of each component is necessary to respond specifically to its cognate signal for dissociation from the complex.

The intrinsic instability of the complex-forming ARSs and AIMPs could be the point at which post-translational modification acts. Although the artificial knock-down experiments revealed the structural interdependence of the components, depletion of components from the complex may also occur in physiological conditions due to dissociation as a result of post-translational modification. For instance, the level of EPRS is severely affected by the absence of its interacting partner, IRS, and of the AIMPs (Figs. 1 and 2), implying that it is intrinsically unstable in isolation. However, EPRS is dissociated from the complex upon treatment with IFN-γ and forms a new complex called GAIT (IFN-γ-activated inhibitor of translation) with ribosomal subunit L13 and GAPDH (3). This shuttling of EPRS between the two complexes is controlled by IFN-γ-dependent phosphorylation of EPRS. Thus, the multifunctionality of the complex-forming ARSs may be regulated by their post-translational modification, which controls their molecular interactions, intrinsic turnover rate, or even cellular localization.

The components of functional macromolecular complexes can be grouped into several categories with respect to how their interactions affect their turnover rates. They can be mutually and equally dependent on each other. Alternatively, complex formation may not affect the stability of the components and be required only for their cellular localization. Third, the components may take up distinct positions in the complex, such as scaffold, master or subordinate, thereby forming a hierarchical network. Our results reveal that the multi-tRNA synthetase complex belongs to the latter category. More specifically, most of the complex-forming ARSs and AIMPs are intrinsically unstable in isolation and are assembled to increase their stability. Second, the components are distinct in terms of their stability. Third, KRS is the least dependent on the other enzymatic components but the most important for the integrity of the complex. Although it is not clear why most of the complex-forming ARSs and their cofactors are unstable in isolation, their instability may provide a "failsafe system" to prevent abortive protein synthesis or undesired or uncontrolled signal transduction. When one or more of the components are dissociated or depleted from the complex, the resulting subcomplex may be inactive in protein synthesis, or the rest of the components may disintegrate and trigger undesirable signaling unless they are destroyed. Thus, it may be safer to degrade the other compo-

nents than to maintain the abortive subcomplex. The linkage patterns in this multifunctional complex may provide insight into the structural organization of other multiprotein complexes that act in various signaling pathways and regulatory processes.

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