An interdomain bridge influences RNA binding of the human La protein

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La proteins are RNA chaperones that perform various functions depending on distinct RNA-binding modes and their subcellular localization. In the nucleus, they help process UUU-3’OH–tailed nascent RNA pol III transcriptions, such as pre-tRNAs, whereas in the cytoplasm they contribute to translation of poly(A)-tailed mRNAs. La accumulation in the nucleus and cytoplasm is controlled by several trafficking elements, including a conserved nuclear localization signal in the extreme C terminus and a nuclear retention element (NRE) in the RNA recognition motif 2 (RRM2) domain. Previous findings indicate that cytoplasmic export of La due to mutation of the NRE can be suppressed by mutations in RRM1, but the mechanism by which the RRM1 and RRM2 domains functionally cooperate is poorly understood. In this work, we use electromobility shift assays (EMSA) to show that mutations in the NRE and RRM1 affect binding of human La to pre-tRNAs but not UUU-3’OH or poly(A) sequences, and we present compensatory mutagenesis data supporting a direct interaction between the RRM1 and RRM2 domains. Moreover, we use collision-induced unfolding and time-resolved hydrogen–deuterium exchange MS analyses to study the conformational dynamics that occur when this interaction is intact or disrupted. Our results suggest that the intracellular distribution of La may be linked to its RNA-binding modes and provide the first evidence for a direct protein–protein interdomain interaction in La proteins.

This article contains Figs. S1–S5.

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cessing in *Schizosaccharomyces pombe* cells occur, in which pre-tRNAs retain their 5′ leaders and 3′ trailers (23, 27). Cytosplasmic accumulation upon mutation of the NRE was demonstrated to occur due to aberrant nuclear export of La and not due to a lack of nuclear import, as nuclear accumulation of hLa or Sla1p NRE mutants and consequent restoration of normal pre-tRNA processing occurs upon treating cells with the Crm1-dependent nuclear export inhibitor leptomycin B (23, 27). Interestingly, nuclear accumulation and normal pre-tRNA processing could also be rescued through point mutations to the α1 helix of RRM1 (Ref. 27; helix numbering as in Ref. 28). Based on the capacity of RRM1-α1 mutations to restore nuclear accumulation of NRE mutants, it was hypothesized that the RRM1 domain may harbor a nuclear export activity that is functionally masked by the NRE associated with RRM2 in hLa and C-terminal to RRM1 in Sla1p (27). However, amino acids in RRM1 whose mutation restores nuclear accumulation of La dNRE do not conform to any Crm1 nuclear export sequence (NES) consensus. Despite the apparent functional link between the RRM1-α1 and RRM2-α3 (NRE) regions, direct evidence for an interaction between these domains is still lacking.

Based on the observation that the RRM1, RRM2, and C-terminal regions of La function in RNA binding outside of UUU-3′OH recognition, we considered the possibility that the altered localization of La variants harboring mutations to their RRM1-α1 helix and/or RRM2-α3 helix could be related to an altered capacity of such mutants to engage La RNA targets. We therefore tested hLa and hLa–dNRE for altered capacity to bind a UUU-3′OH–containing substrate (U10), a poly(A) substrate (A20), and a pre-tRNA substrate. We also tested for a direct interaction between the RRM1-α1 helix and RRM2-α3 helix by making compensatory mutations to these two regions and assessing these mutants for their ability to bind these same RNAs. Finally, we tested hLa and various mutants for changes in their structural dynamics using collision-induced unfolding (CIU) and time-resolved electrospray ionization hydrogen-deuterium exchange (TRESI-HDX) in the presence and absence of RNA to better understand the nature of potential interactions between RRM1-α1 and RRM2-α3 and how these are affected by the presence of a ligand. Our data demonstrate that mutations to the RRM1-α1 helix and RRM2-α3 helix indeed affect the association of hLa with RNA and provide evidence of a direct, physical interaction between the RRM1-α1 helix and RRM2-α3 helix. Altered engagement of NRE mutants with RNA may thus be related to their role in subcellular localization.

## RESULTS

### Mutation to the nuclear retention element results in altered RNA binding to pre-tRNA targets

It has been previously demonstrated that hLa engages a variety of RNA targets using various RNA-binding modes (2, 3, 6, 7, 22, 29–31). La engages RNA polymerase III targets with high affinity, binding terminal uridylylates (UUU-3′OH) using conserved amino acids on the La motif combined with non-UUU-3′OH–associated contacts via the canonical RNA-binding surface of RRM1 (2, 3). We have also recently demonstrated that La binds polyadenylate sequences with high affinity provided the poly(A) sequence is lengthy and that this RNA-binding mode is at least partially independent from the previously characterized UUU-3′OH–dependent RNA-binding mode (22). Other work has also demonstrated that La binds structured RNAs that lack UUU-3′OH such as the domain IV region of the hepatitis C viral RNA (7). This alternative binding mode relies on the La motif, RRM1, and RRM2 regions and does not display any apparent sequence specificity, even if it requires (a) a hairpin and (b) either a 5′ or 3′ single-stranded extension. Finally, we have recently demonstrated that the unstructured CTD of hLa also cooperates with RRM2 in the engagement of single-stranded and dsRNA targets, also without any apparent sequence specificity (6).

Based on the breadth of demonstrated RNA-binding modes for La proteins, we considered whether mutation of the NRE may alter these patterns. To test this hypothesis, we performed electromobility shift assays (EMSAs) comparing WT hLa RNA binding to an hLa mutant with the nuclear retention element deleted (hLa dNRE) for the RNA targets U10, U20, A20, and a leader, intron, and trailer (including a UUU-3′OH, total length 108 nucleotides) containing pre-tRNA substrate (pre-tRNA AlaCGC) (Fig. 1C). We observed similar binding between the hLa and hLa dNRE for the U10 substrate (K_{rel} hLa dNRE/hLa for U10 = 1.5), consistent with previous work showing that UUU-3′OH–dependent binding relieves primarily upon the La motif and RRM1 contained within the La module. We observed a slight increase in affinity by hLa for the U20 substrate relative to the U10 substrate (K_{rel} hLa U10/hLa U20 = 2.1), consistent with previous work (22). However, this enhanced binding to U20 was lost in the hLa dNRE mutant (K_{rel} hLa dNRE U10/hLa dNRE U20 = 1.1), consistent with regions C-terminal to RRM2 contributing to binding of lengthy, single-stranded RNAs (6). We did not observe any substantial defect in hLa dNRE binding to A20 (K_{rel} hLa dNRE/hLa for A20 = 1.1; Fig. 1C), suggesting that the NRE does not contribute significantly to this binding mode, also consistent with previous work (22). However, we did observe a more substantial decrease in binding of hLa dNRE to the pre-tRNA substrate (K_{rel} hLa dNRE/hLa for pre-tRNA = 3.7). We also observed an altered pattern of pre-tRNA substrate supershifting in which a prominent hLa–pre-tRNA ribonucleoprotein (RNP) species (marked with “2” on the hLa EMSA; initial binding event indicated as “1”) was absent in the hLa dNRE EMSA. These supershifts represent multiple La proteins binding the same RNA substrate when the RNA target is sufficiently large so as to accommodate multiple La proteins (1). Because
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the primary pre-tRNA–binding event includes the UUU-3′OH trailer (29) but subsequent La-binding events can no longer bind the now occupied 3′ end, altered supershifting in the hLa dNRE mutant suggests that this variant has an altered RNA-binding capacity in UUU-3′OH–independent binding modes. This is also consistent with the observed length-associated difference in U10 and U20 binding for the hLa dNRE mutant.

To further investigate differences in RNA binding between WT and hLa dNRE, we performed EMSA with three other RNA substrates. The first was A17U3 (Fig. S1), which would combine the UUU-3′OH–dependent RNA-binding mode with the length-dependent adenylate-binding mode. The others were previously characterized substrates (7) derived from a stem-loop from the domain IV region of hepatitis C virus IRES; one of these included a four-nucleotide 5′ single-stranded sequence extending from the stem-loop (5′ss-SL; Fig. 1) and the other a six-nucleotide 3′ single-stranded sequence extending from the stem-loop (SL-3′ss; Fig. S1). Similar to U10 and A20, we found that hLa and hLa dNRE had similar affinities for A17U3 (Fig. S1), consistent with the nuclear retention element not playing a major role in the binding of either of these substrates. Although both hLa and hLa dNRE bound the SL-3′ss substrate similarly, the hLa dNRE mutant had ~2.3-fold lower binding to the SL-5′ss substrate, more similar to the pre-tRNA. These data suggest that the NRE may function in the binding of single-stranded RNAs that are 5′ to structured RNA, as the pre-tRNA substrate tested also has a 5′ single-stranded region in the form of the leader. These data are reminiscent of previous work linking the region immediately C-terminal to the NRE, the short-basic motif, to the binding and processing of pre-tRNA 5′ leader sequences (33), in which mutation of this region resulted in enhanced access of a pre-tRNA 5′ leader sequence to RNase P. In sum, these data suggest that the NRE functions in binding to UUU-3′OH–independent and poly(A)-independent RNA contacts.

Compensatory mutations in RRM1 restore altered RNA-binding patterns observed for nuclear retention element mutants

Previous work has demonstrated that point mutation of the RRM2-α3 NRE amino acids hLa K316A/K317A, or the equivalent S. pombe La (Sla1p) amino acids K234A/K235A, results in an accumulation of La in the cytoplasm and accompanying defects in La-dependent pre-tRNA processing similar to the deletion of the entire nuclear retention element (hLa(d316–332)) (27). Interestingly, further mutation of the RRM1-α1 amino acids hLa E132A/D133A, or the equivalent Sla1p amino acids E177A/E178A, resulted in restoration of La nuclear localization and rescue of La-dependent pre-tRNA processing. These regions were thus hypothesized to functionally communicate in some way, although evidence for a direct interaction between the RRM2-α3 and RRM1-α1 regions was lacking.

Our EMSA data were suggestive that deletion of the NRE might result in the removal of a region of hLa that makes direct contacts to RNA and as a consequence results in impaired binding for some substrates. Alternatively or in addition, we considered the possibility that the hLa dNRE–associated defects in RNA binding could be the result of the loss of an interdomain RRM1-α1–RRM2-α3 interaction that may be important for UUU-3′OH–independent RNA binding. To attempt to differentiate these hypotheses, we made charge swap mutations to either the RRM2-α3 nuclear retention element (hLa K316E/K317E) or the hypothesized interaction site in RRM-α1 (hLa E132K/D133K), and we then combined these mutations such that an interdomain salt bridge between RRM2-α3 and RRM-α1 might thus be restored (hLa E132K/D133K/K316E/K317E). We then tested these mutants for their capacity to bind a variety of RNA substrates.

Similar to hLa dNRE, we observed no substantial differences in hLa E132K/D133K, hLa K316E/K317E, or hLa E132K/D133K/K316E/K317E binding to U10 or A20 (Fig. 2). Binding to the pre-tRNA Ala CGC target, however, was substantially different across the various mutants. First, all three mutants showed decreased affinity for the pre-tRNA Ala CGC target compared with WT hLa, with hLa K316E/K317E having the lowest affinity among the point mutants, consistent with these regions having a role in UUU-3′OH–independent binding. Both the hLa E132K/D133K and hLa K316E/K317E mutants also displayed an altered RNP banding pattern relative to WT, with hLa E132K/D133K having impaired formation of the species also observed as lacking for hLa dNRE (“2”) and hLa K316E/K317E having impaired formation of both this species (“2”) and the 1:1 complex (“1”). Importantly, the salt bridge compensatory mutant hLa E132K/D133K/K316E/K317E displayed a partial rescue of affinity relative to hLa K316E/K317E, as well as a supershifting pattern more similar to that observed in WT hLa. The restorative partial rescue in the pre-tRNA Ala CGC binding pattern through the combination of the disruptive RRM1 and NRE mutations represents strong evidence that these amino acids indeed make an interdomain protein–protein interaction that impacts how La makes some UUU-3′OH–independent contacts. Notably, the two single-site mutations were not equivalent in their affinity for the pre-tRNA substrate, with hLa E132K/D133K binding more avidly than hLa K316E/K317E, as well as having an RNP-binding pattern more similar to WT hLa. These data suggest that whereas the Glu-132/Asp-133 and Lys-316/Lys-317 region may have an additional role in RNA binding, perhaps through an additional direct contact to RNA. In this model, the charge swap hLa K316E/K317E mutation would result in impaired binding to the main body of the pre-tRNA, because this mutation has no effect on U10 binding, as well as an impaired interdomain RRM1-α1 RRM2-α3 interaction, which affects La supershifting pre-tRNAs via UUU-3′OH–independent contacts. The combined hLa E132K/D133K/K316E/K317E mutation would then rescue the interdomain bridge and supershifting pattern for UUU-3′OH-independent binding events even if the Lys-316/Lys-317 RNA contact site is still impaired, thus accounting for only the partial rescue in affinity relative to WT.

Mutations to the nuclear retention element influence the ability of La to discriminate uridylate versus adenylate targets

We have previously demonstrated that hLa binds UUU-3′OH and lengthy adenylate sequences via binding modes that
are at least partially distinct, in that a U10 RNA is generally ineffective in causing hLa to dissociate from an A20 substrate (22). Given our data in support of an interdomain contact between RRM1-α1 and RRM2-α3, we hypothesized that breaking this interdomain contact may alter the capacity of La to discriminate a UUU-3′-OH–containing substrate from an adenylate-containing substrate and decided to test this using a competition EMSA. In these assays, A20 was radioactively labeled, and we determined a sufficient amount of protein so as to achieve 95% binding (2 μM, Fig. 3, lanes NC). We then mixed increasing amounts of cold A20 or U10 RNA (0.5–32 μM) and then added this amount of protein to these reactions to test the ability of the cold RNAs to displace radiolabeled A20 from hLa or hLa mutants. Notably, free A20 continues to migrate as a monomer with increasing concentrations of U10, consistent with a lack of A20 and U10 dimerization at the temperature (37 °C) of the assay as we have described previously (22). For WT hLa, we observed that increasing amounts of cold U10 were poorly able to result in release of radiolabeled A20, consistent with the poor capacity of these substrates to compete with one another on La, as we have observed previously (22). Deletion or point mutation (hLa K316E/K317E) of the nuclear retention element, however, resulted in a greater dose-dependent release of the A20 RNA upon addition of increasing amounts of U10 (Fig. 3E). Interestingly, the K316E/K317E point mutation was more prone to U10 competition for A20 binding than the hLa dNRE mutant, possibly due to this charge-swap mutation repulsing an interdomain arrangement that stabilizes A20 binding. Restoration of the hypothesized RRM1/RRM2-NRE interdomain contact via the hLa E132K/D133K/K316E/K317E mutant resulted in a partial rescue of the capacity of La to discriminate the UUU-3′-OH– and adenylate-binding modes. Although these experiments do not address the specific mechanisms by which La binds 3′UUU-OH versus poly(A), these data reinforce the idea that UUU-3′-OH– and adenylate-binding modes are distinct and suggest that a RRM1-α1/RRM2-α3 bridge supports a conformation in which La associates stably with lengthy adenylate sequences.

Collision-induced unfolding reveals structural differences in RNA binding between hLa and the NRE-deficient mutant

To study the changes in conformational stability for hLa and hLa dNRE upon RNA binding, we employed CIU. CIU uses ion mobility-mass spectrometry (IM-MS) to measure gas-phase conformational transitions in proteins in the presence and absence of ligands (34–36). IM-MS is a technique used to separate ions based on their size, shape, and charge, effectively a gas-phase analogue of electrophoresis. For globular proteins electrosprayed from “native-like” solvents, IM-MS–based size measurements generally correlate well with the expected size...
from X-ray or NMR structures, suggesting that proteins largely retain their native conformations through transfer into the gas phase (37). In the CIU experiment, native protein ions are subjected to increasing collision energies (voltages), which result in unfolding of the protein and an accompanying increase in size, immediately prior to IM-MS analysis. In the gas phase, these unfolding transitions, which can correspond to unfolding of domains, subdomains, or loss of interactions between domains, often occur at lower energy than loss of ligand, allowing CIU to act as a measurement of the impact of ligand binding on overall conformational stability in the gas phase (34).

For hLa, three major protein conformations with two stages of unfolding were observed in all CIU profiles; however, the energy needed to induce these changes varied for each sample (Fig. 4). In hLa, the native conformation remained intact until 35 V, where a distinct transition is observed corresponding to the adoption of a larger, partially unfolded state (Fig. 4A). The partially folded state is exclusively populated from 45 to 95 V, whereupon the beginnings of a second unfolding transition are observed (the collision energy was not increased beyond 100 V as this induced ligand loss and fragmentation of the peptide backbone). Likewise, in hLa dNRE, there was a transition to the first unfolded state at 35 V; however, in this case there was an almost immediate transition to the second unfolded state (Fig. 4B). The partially unfolded states adopted by hLa and hLa dNRE have the same drift times, suggesting that they are identical, at least in the sense of having the same sizes relative to the folded state. These data are consistent with a contact that stabilizes the native fold of the ligand-free protein that is lost upon deletion of the NRE.

To determine how RNA binding would impact the stability of hLa, a U10 RNA ligand was used (only the U10 RNA–hLa

![Figure 3. Competition experiments show that mutation of the nuclear retention element results in U10 competing better for A20 binding relative to WT hLa.](image-url)

A, competition experiment in which increasing amounts of cold U10 (left) or A20 (right) are tested for ability to displace radiolabeled A20 from hLa. NP, no protein added; NC, no cold competitor added. B–D, same as for A, but testing for hLa dNRE (B), hLa K316E/K317E (C), and hLa E132K/D133K/K316E/K317E (D). E, graphical representation of capacity of cold U10 to displace radiolabeled A20 from various tested mutants. Error bars, standard deviation.
complex was retained well enough through ESI to be subjected to CIU analysis). Consistent with a specific interaction, binding of U10 resulted in an increase in gas-phase conformational stability for the compact protein, in that a higher collision energy (40 V) was required to induce the transition to the first partially unfolded state (Fig. 4B). Interestingly, the energy required for transition to the second unfolded state was drastically reduced to 65 V. One possible explanation for this observation is that the second transition is associated with loss of an intramolecular structural interaction that is eliminated or changed by U10 binding. When U10 was allowed to bind to hLa dNRE, there was an increase in stability in both transition states, resulting in a profile that is more similar to that of the U10-bound hLa (Fig. 4C). This result is in agreement with the EMSA data showing that WT hLa and hLa dNRE have similar affinities for U10 RNA.

Taken together, the CIU data provide evidence for a model of the hLa–U10 RNA interaction with the following features. (i) U10 binds specifically to WT and hLa dNRE in a manner that stabilizes the natively folded state. Consistent with previous work, we hypothesize that the U10-bound natively folded state fixes and stabilizes the orientation of the La motif with RRM1 with respect to one another. (ii) U10 RNA binding disrupts an intramolecular structural interaction that is strong in hLa, but absent in hLa dNRE. This suggests an intramolecular contact involving the NRE helix (which we hypothesize is an RRM-α1/RRM2-α3 bridge) is incompatible with U10 binding and that this bridge breaks to engage a UUU-3′OH substrate. (iii) Binding of U10 RNA to hLa dNRE increases the energy required to induce the second unfolding transition to be more similar to that observed for WT hLa in the presence of U10, indicating that U10 binding may partially stabilize the first partially unfolded state even as it breaks the interdomain contact. This suggests that U10 may make residual or minor contacts in the region around the NRE. Because the hLa dNRE mutant showed lower affinity than WT hLa for the U20 substrate, but this mutant bound U10 more similarly to hLa, we hypothesize that the U10 substrate may make some contacts to the NRE region associated with the second unfolded transition and may partially stabilize this state during collision-induced unfolding.

**Time-resolved hydrogen deuterium exchange on hLa and hLa dNRE**

To further examine the structural and dynamic changes associated with RNA binding to hLa, we used HDX-MS (38, 39). This approach provides more structural detail than CIU and specifically measures the impact of ligand binding in solution. In this type of comparative HDX study, backbone amide deuterium uptake in the free protein is compared with backbone amide deuterium uptake in the protein–RNA complex to reveal changes in the hydrogen bonding network that maintains secondary and tertiary structure. To visualize these changes, uptake differences are mapped onto native PDB structures, where blue and red represent significant decreases and increases in deuterium uptake, respectively, in the protein–RNA complex compared with the unbound protein. Sequence coverage of peptides detected by HDX is provided in Fig. S2, and sample raw and kinetic data for HDX are provided in Figs. S3–S5.

When hLa binds to a U10 RNA ligand, significant decreases in deuterium uptake are observed in the NTD (Fig. 5A). This is consistent with what is commonly observed in comparative HDX experiments when ligand binding induces a “tightening” of the hydrogen bond network and reduces solvent access at and immediately around the binding site. Decreases in this region can therefore be attributed to U10 binding at the NTD as expected based on previous work. One region of the NTD that exhibits no change in uptake upon U10 binding is the RRM1-α1 helix. This may result from a lack of stabilizing interactions in this region, but it is more likely the consequence of a strongly stabilizing interdomain interaction being replaced by an equally stabilizing ligand interaction in the vicinity of this helix upon binding, which is in agreement with the CIU-derived model. It also consistent with the RRM1-α1 helix being the interdomain binding partner for the NRE in unbound hLa.
In contrast to the largely “rigidified” NTD, several regions in the CTD exhibited significant increases in deuterium uptake upon U10 binding, most notably in the NRE RRM2-α3 helix (Fig. 5A). This result is also entirely in agreement with the model proposed from the CIU data, suggesting that when U10 binds, the NRE RRM2-α3 helix is released from a stabilizing interaction, presumably as it is displaced from near the U10-binding site. In the CIU data, this displacement corresponds to the second unfolding transition, which is affected by U10 binding and strongly affected by removal of the NRE. Taken together, the HDX profiles for the RRM1-α1 and RRM2-α3 helices in the presence and absence of ligand indicate that these regions are in contact in the free protein and dissociated upon U10 RNA complexation.

When bound to A20, hLa showed decreased deuterium uptake levels throughout the protein, most notably at the RRM2-α3 NRE helix (Fig. 5B). This result is also entirely in agreement with the model proposed from the CIU data, suggesting that when U10 binds, the NRE RRM2-α3 helix is released from a stabilizing interaction, presumably as it is displaced from near the U10-binding site. In the CIU data, this displacement corresponds to the second unfolding transition, which is affected by U10 binding and strongly affected by removal of the NRE. Taken together, the HDX profiles for the RRM1-α1 and RRM2-α3 helices in the presence and absence of ligand indicate that these regions are in contact in the free protein and dissociated upon U10 RNA complexation.

When bound to A20, hLa showed decreased deuterium uptake levels throughout the protein, most notably at the RRM2-α3 NRE helix and the RRM1-α1 helix (Fig. 5B). This substantial difference from the U10-bound profile is likely because A20 binding results in A20-associated protections as well as a stabilization of the interdomain contacts observed in the vacant protein. Alternatively, when the interdomain interaction in the free protein is eliminated (i.e. in hLa dNRE), the U10- and A20-bound profiles are essentially identical (Fig. 5, D and E). Based on this result, we can predict that dNRE hLa will exhibit decreased discrimination between U10 and A20 ligands, which is reflected in the competition EMSAs described earlier.

**Time-resolved hydrogen deuterium exchange on the RRM1-α1 and RRM2-α3 charge swap mutations**

To acquire more definitive evidence of an interaction between the RRM1-α1 and RRM2-α3 helices, we applied HDX to the disruptive and compensatory point mutations described earlier (K316E/K317E and E132K/D133K/K316E/K317E, respectively). The prediction for hLa K316E/K317E would be that it should not exhibit the increase in deuterium uptake in the NRE associated with U10 binding (Fig. 5A) because there is no interdomain interaction to release. A comparison of Figs. 5A and 6B indicates that this is the case, with the crucial similarity being decreased relative to uptake in the NRE helix. Similarly, when the interdomain interaction is rescued through the compensatory mutation, the HDX uptake difference profile should resemble that associated with U10 binding to WT hLa. Based on a comparison of Figs. 5A and 6E, this similarity is also clear, with the characteristic increase in uptake in the NRE helix present. Similarly, the WT protein and compensatory point-mutated La mutant have similar HDX profiles upon A20 binding, although this is not the case in the single disruption of the NRE (hLa K316E/K317E), which in this case appears more similar to WT hLa binding to U10.

**Discussion**

La proteins have been associated with a variety of RNA ligands and binding modes. The most extensively studied binding mode is La binding to UUU-3’OH, commonly found at the end of nascent RNA polymerase III transcripts, which is the only binding mode for which high-resolution RNA–protein complexes are available. La proteins have also been implicated in the sequence-specific and length-dependent binding of poly(A), as well to the relatively nonspecific binding of structured RNA substrates such as stem-loops or the main body of a pre-tRNA. In the absence of RNA binding, it has been hypothesized that the La motif and RRM1 are not in a fixed orientation with respect to one another (2), and it has been hypothesized that the relative conformational freedom between the LAM and RRM1 may be important for the capacity of La to engage a variety of RNA substrates. It is expected that this interdomain flexibility has made the structural determination of RNA-bind-
ing modes in which more than one domain is used quite challenging.

Previous work identified the nuclear retention element as a region associated with RRM2 that is important for La intracellular localization. A high-resolution structure of RRM2, including the NRE but in the absence of RNA, revealed that the NRE adopts an extended \( \alpha \)-helical structure that obscures the canonical RNA-binding surface of the RRM (25). This unusual arrangement (named the \( x \)RRM motif (40)) has subsequently been observed in the RRM2 of the La-related protein p65 (26), and both the NRE region of La and the equivalent region of p65 have been implicated in RNA binding (6, 26). However, the basis by which this region contributes the nucleo-cytoplasmic trafficking of La in human and fission yeast cells is not well understood.

Interestingly, the defect in La nuclear accumulation NRE mutants can be suppressed with mutations to the RRM1 region, and it was hypothesized that the NRE may mask a region important for La nuclear export elements in this region (27). Although export of NRE mutants has been demonstrated to be Crm1-dependent, regions on RRM1 important for this nuclear export activity (including RRM1-\( \alpha \)-1 amino acids Glu-132 and Asp-133) do not conform to the Crm1 consensus sequence, nor has any direct protein–protein interaction been demonstrated between La and Crm1. Therefore, it is not apparent that the region (or regions) on RRM1 important for La nuclear export constitute a \textit{bona fide} NES. In sum, the relationship between the NRE, RRM1, and the interplay of these regions in the control of La intracellular trafficking is not well understood.

In this work, we strengthen previous evidence that the NRE on RRM2-\( \alpha \)-3 and RRM1-\( \alpha \)-1 amino acids Glu-132 and Asp-133 make a direct protein–protein contact through the use of compensatory mutagenesis. EMSA analyses of La and La mutant binding to a variety of substrates, as well as CIU and TRESI-HDX–MS based analysis, strongly converge on a unified model in which the NRE and the \( \alpha \)1 helix region of RRM1, which includes amino acids Glu-132 and Asp-133, make a direct protein–protein interaction that either breaks or forms depending on the identity of the bound RNA ligand. Notably, disruption or compensatory restoration of the NRE–RRM1 bridge has little effect on the affinity of La for U10 or A20 substrates, even if our data are most consistent with the bridge being broken during binding to U10 and being intact during binding of A20.

Disruption of the NRE is not without consequences for binding to RNA substrates, however. We also demonstrate that dNRE binding to a full pre-tRNA substrate or, to a lesser extent, a U20 homopolymer is impaired relative to WT, which is consistent with other data linking RRM2 to the binding of structured RNA substrates in the absence of sequence specificity. This impaired binding is mirrored in the loss of supershifting species in the EMSA analysis upon mutation of the NRE, because supershifts are binding events secondary to the primary binding event, this is also indicative of defects in RNA binding that do not utilize the UUU-3\( ^{\text{OH}} \)–dependent binding mode. Because our binding to the pre-tRNA substrate is impaired in dNRE mutants, and our CIU and TRESI-HDX data indicate that the NRE RRM2-\( \alpha \)-3/RRM1-\( \alpha \)-1 bridge is not formed during uridylate binding, we hypothesize that the breaking of the bridge during La binding to pre-tRNAs (which uses the uridylate binding mode) makes the basic amino acids of the NRE accessible for making contacts to the pre-tRNA

![Figure 6. Differences in deuterium uptake mapped onto the structures obtained from PDB (codes 1OWX and 1YTY).](image-url)

A) hLa K316E/K317E
B) hLa K316E/K317E +U10
C) hLa K316E/K317E +A20
D) hLa D132K/E133K/K316E/K317E
E) hLa D132K/E133K/K316E/K317E +U10
F) hLa D132K/E133K/K316E/K317E +A20

Areas of the protein with decreased, increased, or no change in deuterium uptake are highlighted in blue, red, and tan, respectively. The areas colored gray means there were no observed peptides from that area.
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body/5’ leader. This model also provides a potential explanation for why the K316E/K317E mutations have greater defects in tRNA binding relative to the E132K/D133K single mutations, as well as for why the compensatory mutagenesis (K316E/K317E/E132K/D133K) only results in a partial restoration of pre-tRNA binding.

In sum, we present a variety of experimental data that are consistent with a direct protein–protein interaction between the RRM1 and NRE region of RRM2 of the human La protein. This is, to our knowledge, the first experimental evidence for an interdomain protein–protein contact in a genuine La protein. We furthermore demonstrate data consistent with the disruption and restoration of this contact depending on the identity of RNA substrate bound to La: this bridge is (or can be) intact in the vacant protein and possibly when bound to A20, whereas it is broken during uridylation and pre-tRNA binding. Although the data presented are consistent with this model, a firm interpretation of these potential interdomain contacts and how they factor in RNA binding will almost certainly require one or more relevant high-resolution structures. One other outstanding issue is how this interdomain contact functions in La intracellular localization. One intriguing hypothesis is that altered La–RNA-binding modes in the context of NRE mutants alters the complement of La-bound RNAs such that the (potentially Crm1-dependent) intracellular transport of these transcripts indirectly influences whether La localizes to the nucleus or cytoplasm. Future work will investigate this possibility further.

Experimental procedures

EMSAs

All tested RNAs except the pre-tRNA substrate were chemically synthesized (IDT) and 5’-end-labeled used γ-[32P]ATP and T4 PNK (New England Biolabs). Pre-tRNA Ala CGC (S. pombe, chromosome I nucleotide 4796977) was synthesized by SP6 in vitro transcription (SP6 Megascript transcription kit, Ambion, AM1330) using γ-[32P]ATP and a PCR-generated DNA template containing an 8-nucleotide 5’ leader, the intron, and an 18-nucleotide 3’ trailer (up to the endogenous transcription termination site, including five uridylates). All radiolabeled RNAs were PAGE-purified from TBE-urea gels after synthesis. Recombinant His-tagged human La (41) or hLa point mutants (generated by QuikChange; Agilent) were purified from E. coli (generated by QuikChange; Agilent) were purified from E. coli (EScherichia coli) first over a Ni2+ column (His-Trap, GE Healthcare) and then a heparin column (Hi-Trap, GE Healthcare). Proteins were then concentrated and quantitated via Bradford and SDS-PAGE, and A260/A280 ratios were taken to confirm purified proteins were free from contaminating RNAs that might have copurified from E. coli. EMSAs were performed as described (29). Briefly, 3000 cpm (~0.1 nm) of various RNA substrates were incubated with various concentrations of recombinant human La or human La mutants in a 20-μl reaction containing 1× EMSA buffer (20 mM Tris, pH 7.6, 100 mM KCl, 1 mM EDTA, and 5 mM β-mercaptoethanol). RNAs were initially slow-cooled from 95 °C to room temperature and then incubated with protein at 37 °C for 20 min. Complexes were resolved on 10% (w/v) polyacrylamide nondenaturing gels at 4 °C at 100 V. Supershifts were treated as supplementary binding events to the primary binding event, and binding curves were fit using a nonlinear specific binding curve fitting program (GraphPad, Prism). Kd values were approximated as the concentration of protein at which half of the RNA substrate was bound. EMSAs were performed at least in duplicate, and representative gels are provided.

For competition experiments, radiolabeled A20 RNA was mixed with 50 ng/μl cold SL-3’s and the indicated amounts of cold U10 RNA in 1× EMSA buffer, after which 2 μM recombinant hLa was added and incubated for 20 min at 37 °C, prior to separation on native PAGE as described above.

Collision-induced unfolding

Samples were buffer-exchanged into 250 mM ammonium acetate (C2H5O2NH4, pH 8) by ultrafiltration and analyzed using a quadrupole–ion mobility–time-of-flight mass spectrometer instrument (Synapt G2-S HDMS, Waters). Protein ions were generated using an ESI source in positive mode. The source operated with a capillary voltage of 3.0 kV and sampling cone of 100 V. To generate ion mobility separation, the traveling wave ion mobility cell operated at a pressure of 3.79 mbar and a series of DC voltage waves (wave height of 40 V with a velocity of 652 m/s) were used. The instrument operated over a mass-to-charge range of 1000–5000 at a pressure of 1.09 × 10−6 mbar.

To perform CIU experiments, collision energy was applied to the ions in the ion trap located prior to the ion mobility cell. The collision energy voltage was ramped from 10 to 100 V in 5-V increments to construct a CIU fingerprint. The CIU fingerprints were generated using PULSAR (42).

Microfluidic chip fabrication

A microfluidic device was made as described previously (6, 43) with some modifications. A proteolytic chamber and capillary channels were etched onto a polymethyl methacrylate (PMMA) substrate (dimensions: 0.83 × 0.25 × 0.1778) using a VersaLaser engraver (Universal Laser, Scottsdale, AZ). Another blank PMMA substrate with the same dimensions was used as a seal, and the two were welded together using acrylic glue. A glass capillary with an outer diameter of 151 μm and a 33-gauge stainless steel metal capillary were screwed into the chip via double-winged nuts, used as the input and output channel, respectively. The output channel also served as the ESI capillary.

The time-resolved mixer was made by inserting a glass capillary (outer diameter = 152.0 μm) into a metal capillary (inner diameter = 177.8 μm) creating an intercapillary space of ~26 μm. The end of the glass capillary was sealed, and a notch was cut 2 mm from the sealed end using the VersaLaser engraver. For reaction quenching, a T-mixer with a dead volume of 51 nl was used to mix 10% glacial acetic acid with deuterated protein. The device was placed at the front end of a modified NanoSpray platform on a quadrupole–ion mobility–time-of-flight mass spectrometer instrument (Synapt G2-S HDMS, Waters).

TRESI-HDX

The HDX reaction took place inside the TRESI mixer in which gas-tight Hamilton syringes were used to deliver the re-
agents through the capillaries using syringe infusion pumps (Harvard Apparatus). 5 μM hLa in 250 μM ammonium acetate (C₂H₃O₂NH₄, pH 8) flowed through the inner capillary at 2 μl/min while deuterium flowed through the outer capillary at 2 μl/min. The total flow rate in the TRESI mixer (4 μl), the inter-capillary space, and the inner capillary pulled back at 1 mm created a reaction volume of 25.8 nl and an HDX reaction time of 0.59 s. The instrument operated at a voltage of 2.25 kV in positive ion mode with a sampling cone voltage of 25 V. The samples were scanned over a mass-to-charge range of 400–1500. The experiment was performed on two biological replicates each having three technical replicates.

A digestion profile was acquired prior to the HDX reaction for the purpose of peptide identification. The ExPASy FindPept on-line server was used to find the possible fragments corresponding to each peptide. Tandem MS was then used to verify the correct identity of each peptide. Data Analysis was carried out using the MS studio software (32) in which the deuterium levels for each peptide in free protein and 1:1 protein–RNA complex was calculated. Differences in deuterium uptake that fell within two σ (95% confidence interval) was considered to be significant. The significant differences were then mapped onto their corresponding PDB structures.

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