Systematic Mutagenesis of the Leucine-rich Repeat (LRR) Domain of CCR4 Reveals Specific Sites for Binding to CAF1 and a Separate Critical Role for the LRR in CCR4 Deadenylase Activity*

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CCR4, a poly(A) deadenylyase of the exonuclease III family, is a component of the multiprotein CCR4-NOT complex of Saccharomyces cerevisiae that is involved in mRNA degradation. CCR4, unlike all other exonuclease III family members, contains a leucine-rich repeat (LRR) motif through which it makes contact to CAF1 and other factors. The LRR residues important in contacting CAF1 were identified by constructing 29 CCR4 mutations encompassing a majority of residues interstitial to the conserved structural residues. Two-hybrid and immunoprecipitation data revealed that physical contact between CAF1 and the LRR is blocked by mutation of just two α-helix/β-helix strand loop residues linking the first and second repeats. In contrast, CAF16, a potential ligand of CCR4, was abrogated in its binding to the LRR by mutations in the N terminus of the second β-strand. The LRR domain was also found to contact the deadenylase domain of CCR4, and deletion of the LRR region completely inhibited CCR4 enzymatic activity. Mutations throughout the β-sheet surface of the LRR, including those that did not specifically interfere with contacts to CAF1 or CAF16, significantly reduced CCR4 deadenylase activity. These results indicate that the CCR4-LRR, in addition to binding to CAF1, plays an essential role in the CCR4 deadenylylase of mRNA.

CCR4 is a poly(A)-specific deadenylylase of the exo-nuclease III family, is a component of the multiprotein CCR4-NOT complex of Saccharomyces cerevisiae (3, 4). As a component of the CCR4-NOT complex of Saccharomyces cerevisiae (5, 6), it functions in the deadenylylation of mRNA poly(A) tails (7, 8), the first step in a major mRNA degradation pathway (9). The CCR4-NOT complex is evolutionarily conserved among eukaryotic organisms. At least six subfamilies are recognized based on the differing lengths and consensus sequences of the repeats; “typical” repeats consist of 20–27 residues (19). X-ray structural data are currently available for the LRR domains of several proteins including porcine and human placental ribonuclease inhibitor (RI) (20), Schizosaccharomyces pombe rna1p (21), the RNA-binding human spliceosomal U2A' (22), the internalin B protein of the bacterium Listeria monocytogenes (23), and the human mRNA export factor TAP (22, 27). Although the number of tandem repeats in these LRR structures is variable, ranging from 5 to 17 repeats, the overall nonglobular topology of each LRR is strongly similar. Each repeat is a structural unit consisting of a highly conserved β-strand packed against a more variable strand, usually helical; these are assembled along a common axis into an arc-shaped structure lined with parallel β-strands along the inner surface, an adjoining asparagine or cysteine loop region, and the α-helix, 310 helix, or extended conformation flanking the outer circumference (26, 28, 29). The solvent-exposed parallel β-sheet lining the inner cleft and the curvature of the structure are general features of the LRR motif.

Several studies point to the nonleucine, interstitial residues of the solvent-exposed β-sheet and the βα-turn regions of the LRR motif as involved in protein and RNA ligand recognition and binding (22, 26, 30–32). However, because each of these studies has targeted only a few of the potential LRR contact points, it is not yet clear whether such limited interactions are universally employed by this diverse protein family to mediate binding.

CCR4 contains a central region of five complete tandem LRRs of 23 amino acid residues in length, plus one partial repeat, which together span residues 335–467 (14). On the basis of the length and consensus sequence of its LRR domain, CCR4 is classified as belonging to the most highly populated or

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1 The abbreviations used are: LRR, leucine-rich repeat; CG, choriocic gonadotropin; GST, glutathione S-transferase; RI, ribonuclease inhibitor.
typical subfamily of LRR proteins (19). Deletion of two or more LRRs of CCR4 results in a nonfunctional protein that is unable to complement a defective ccr4 allele and fails to bind to CAF1 and its other potential ligands (5, 14, 17, 33). The direct physical contact between CCR4 and CAF1 has been established by two-hybrid assays and by immunoprecipitations (5, 6, 15). However, the CCR4 contact to CAF1 and its other potential ligands has only been shown by two-hybrid assays (17), and the physiological significance of these contacts remains to be determined.

We have taken advantage of the small size and relative simplicity of the LRR of CCR4 to mutate systematically the majority of LRR residues predicted to be involved in ligand binding. A total of 29 mutants (47 amino acid alterations) were characterized for altered binding and functional activity. Here we report the contacts that the CCR4-LRR makes to its protein ligands, CAF1 and CAF16. Moreover, we find that the LRR interacts with the C-terminal exonuclease domain of CCR4 and, importantly, is absolutely required for its activity. Mutations across the β-sheet surface, including those that do not interfere with CAF1 binding, significantly reduced CCR4 enzyme activity, whereas mutations in other regions generally had less or no effect on CCR4 activity. These data demonstrate that the relatively small LRR domain of CCR4 is essential both for binding CAF1 and for CCR4 deadenylase function.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions—The following yeast strains were used in this study: EGY188 (MATa his3 leu2 trp1 ura3 LexA-LEU2), EGY191 (MATa his3 leu2 trp1 ura3 LexA-LEU2), EGY188-1a (isogenic to EGY188 except ccr4 mating-type), MD9-7c (MATa adh1–11 his3 trp1 ura3 ccr4–10), and TM4 (isogenic to MD9-7c except trp1:CCR4). Conditions for growth of cultures on minimal medium lacking uracil and histidine or YEP medium (2% Bacto Peptone, 1% yeast extract, 1% galactose, 2% raffinose, and 8 mM caffeine).

RESULTS

CCFR4-LRR Mutations—Site-directed mutagenesis was used to introduce single or double point mutations into the LRR by sequential PCR steps using either of two methods described (36, 37). Briefly, either one or two mutations were introduced into clones, varying in length from 27 to 33 bases, were used in the first round of PCR to amplify template plasmid TM5, which contains the 1.5-kb BamHI–HindIII fragment of S. cerevisiae CCR4, has been described previously (14). MD9 (33) is a LexA-CCR4 fusion plasmid in which full-length CCR4 (residues 1–837) has been fused in-frame with LexA at the EcoRI-BamHI polylinker site of pLexA1 (10). Full-length CCR4-LRR mutants generated by PCR site-directed mutagenesis were cloned into pMD9 as follows. The PCR products were digested with ApaI-BamHI, mutants generated by PCR site-directed mutagenesis were cloned into pMD9 carrying the desired CCR4 mutation was digested with ApaI and Smal. The resulting fragments were gel purified and ligated into the ApaI-Smal site of the TM4 vector. TM4-CCR4-LRR mutant plasmids were first linearized within the TRP1 fragment by digestion with BglII and then for the integrative transformation at the trp1 locus in EGY188-1a. Transformants were initially selected on trp–plates, then subsequently transferred to YD to allow for plasmid loss. Single colonies were grown overnight and lysed; the extracts were then analyzed by SDS-PAGE and anti-CCR4 Western blot analysis to verify the overall structure and integrity of the LRR. To target residues of the LRR, the ternary complex proteins were transformed into strain EGY191, and transformants were mated with EGY188/LexA-CCR4-LRR mutants; yeast extracts were then assayed for CAF1 and CAF16 activity. β-Galactosidase activities reported (in units/mg) are the average of multiple assays of at least four separate transformants. S.E. values were less than 20%.

Immunoprecipitation and Western Blots—The ccr4–null yeast strain, EGY188-1a, containing either wild-type CCR4 or a CCR4-LRR mutation integrated at the chromosomal TRP1 locus, was grown overnight in YEP medium containing 4% glucose. Cells were pelleted, and the whole-cell proteins were extracted in lysis buffer (8 mM K$_2$HPO$_4$, 17 mM KH$_2$PO$_4$, 150 mM KCl, 1 mM sodium pyrophosphate, 1 mM NaF, 1% Nonidet P-40, 10% glycerol, 5 mM MgCl$_2$, 1 mM EDTA, plus protease inhibitors, pH 7.6). The insoluble fraction of the resulting extract was pelleted by centrifugation. A 2-μg aliquot of cleared supernatant was then incubated with 2 μg of polyclonal rabbit anti-CAF1 for 45 min at 4 °C and mixed with 20 μl of protein A-agarose for an additional 30 min. The beads were pelleted by centrifugation in a microcentrifuge and washed three times in 1 ml of the lysis buffer, 1 ml of lysis buffer with 1 μM KCl, and finally 1 ml of Triton X-100 wash buffer. 20 μl of SDS sample buffer was added to the beads, and the beads were boiled for 4 min before being loaded on an 8% SDS-polyacrylamide gel. Western blot analysis was carried out as described using polyclonal anti-NOT1, anti-CAF1, and anti-CCR4 blotting antibodies and alkaline phosphatase detection (10).

RNA Enzyme Assays—CCR4-FLAG protein variants were prepared from the GAL1 promoter (38) in strain EGY188-1a-1 as described previously (1). The RNA substrate 25N+20A was utilized, and the enzyme activities were conducted as described previously (38). Relative enzyme activity was determined over several enzyme concentrations and standardized to that of wild-type CCR4-FLAG. Depending on the enzyme variant, relative activity was quantitated by determining the number of As removed per min or, for those variants having activity similar to wild-type, by determining the rate at which the 20N + 1A substrate was deadenylated (38). Quantitation of CCR4 protein variant abundances was determined by Western analysis using anti-FLAG antibody.

RESULTS

CCFR4-LRR Mutations—A total of 29 CCR4-LRR mutants were generated by PCR mutagenesis, verified by sequencing, and cloned into a yeast expression vector for functional analysis (Fig. 1). The majority of mutants were single or double alanine substitutions of residues lying interstitial to conserved structural residues of the LRR. The conserved residues of the LRR were left intact to minimize alterations that would affect the overall structure and integrity of the LRR. To target residues encompassing all of the repeats efficiently, residues were mutated in pairs wherever possible. Together, these mutations targeted a majority (47 of 81) of the predicted nonconserved residues distributed across the 5.5 LRRs of yeast CCR4 (Fig. 1). For existing glycine or alanine residues the bulky aromatic
residue tyrosine or phenylalanine was substituted. Each of the mutated CCR4 genes was constructed as a LexA-CCR4 fusion to monitor CCR4 interactions with its known ligands rapidly using the two-hybrid assay (10, 17).

All of the mutants except two (K406A/Y407A and C426A/N427A, see “Discussion”) expressed a LexA-CCR4 fusion protein of the predicted size in vivo at levels comparable with wild-type LexA-CCR4 as determined by anti-LexA Western blots (data not shown). Also, all LexA-CCR4-LRR mutants transformed into MD9-7c, a yeast strain bearing the nonfunctional ccr4-10 mutant allele, exhibited complementation of the glycerol temperature-sensitive (ts) and 8 mM caffeine-sensitive phenotypes of ccr4-10 (data not shown) (16).

Each of the stably expressed mutant LexA-CCR4 proteins was initially analyzed for its ability to activate low level transcription from LexA-lacZ reporter, a property characteristic of LexA-CCR4 (33). All LexA-CCR4-LRR mutants retained the ability to activate transcription weakly (data not shown), consistent with the localization of the CCR4 activation domains to regions N-terminal to the LRR (33). We did observe, however, that two alterations, Q429A/F430A and Y409A, resulted in significantly increased levels of lacZ expression (from 10 units/mg of β-galactosidase activity for wild-type to 140 units/mg for Y409A and 180 units/mg for Q429A/F430A). Because these alterations are situated in close proximity to one another in the modeled tertiary structure of the CCR4-LRR (see Figs. 1 and 3), it is possible that these residues may define either a repressor binding site or, more likely, a contact that influences the N-terminal activation domains.

Identification of LRR Mutants Defective in Protein Binding to CAF1 and CAF16—To characterize the effect of the LRR mutations on CCR4 protein binding, each of the LexA-CCR4-LRR mutant proteins was analyzed in two-hybrid interaction assays. LexA-CCR4-LRR mutants were screened for their ability to interact with B42 fusions of the CAF1 and CAF16 proteins. The D357A/F358A alteration reduced by 20-fold the ability of LexA-CCR4 to interact with B42-CAF1 but had no effect on LexA-CCR4 interactions with CAF16 (Fig. 2) or other CCR4 interactions, such as CAF17 (Fig. 2; data not shown). These results suggest that CAF1 contacts the CCR4-LRR primarily through the α-helix/β-strand loop, which links the first and second repeats. In contrast, the T360A/R361A mutation reduced the interaction of CCR4 with CAF16 by a factor of ~16-fold but had no effect on CCR4 interactions with CAF1 (Fig. 2). The T360A/R361A mutations also affected CAF17 interaction (Fig. 2). Because this was the only mutant for which CAF16 binding was severely abrogated leaving the CAF1 contact impaired, CAF16 is likely to contact the CCR4-LRR directly through one or both of these residues.

Several other mutations affected CCR4 two-hybrid interactions with CAF1, CAF16, and CAF17 (Fig. 2). These mutations, Q429A/F430A, D357A, Y409A, and G432F, all of which cluster on the same location across the β-sheet surface of the LRR (Fig. 3), may be affecting a key structural feature of the LRR such as to reduce its two hybrid interactions. Two of these alterations, Y409A and Q429A/F430A, were shown above also to affect LexA-CCR4 activation ability.

LRR Mutations Disrupt the Physical Binding of CAF1 to CCR4—To examine whether the two-hybrid assays reflect physical contacts between CCR4 and CAF1, CCR4-LRR mutant strains were constructed in a ccr4 null strain by the integration of non-LexA fusions of the CCR4-LRR mutants T360A/R361A, D357A/F358A, Q429A/F430A, and G432F into CCR4.

FIG. 1. LRR sequence of yeast CCR4. Residues 336–467 of the LRR sequence of CCR4 are aligned as 5.5 repeats. The consensus sequence of the typical LRR subfamily appears below the CCR4 sequence, and the predicted secondary structural elements are indicated (21). Bold and normal uppercase letters indicate more than 70 and 40% occurrence of a given residue in a certain position, respectively. Lowercase letters indicate 30% or greater occurrence of residues of similar properties: α, a nonpolar residue; β, any residue (21). Boxes indicate residues mutated either together or singly. Residues were changed to alanine in all cases except for A374Y and G432F. Shaded residues resulted in decreased binding of CCR4 to CAF1 (D357A/F358A) or in significant reduction in CCR4 enzymatic activity (see Fig. 6).

FIG. 2. Two-hybrid enzymatic assay data for CCR4-LRR mutants. The ability of LexA-CCR4 mutants to interact with B42 fusion proteins was measured by their ability to activate LexA-lacZ in two-hybrid assays. B42 fusions of CCR4-NOT complex proteins were transformed into strain EGY191, and the transformants were mated with EGY188-34/CCR4-LRR mutants. Yeast extracts from resulting diploids were then assayed for β-galactosidase activity. β-Galactosidase activities reported (in units/mg) represent the average of multiple assays of at least four separate transformants. The S.E. was less than 20%. All assay values for the LRR mutants are plotted as a percentage of two-hybrid values for the interaction of wild-type LexA-CCR4 with CAF1 (2,000 units/mg), CAF16 (570 units/mg), and CAF17 (310 units/mg). All values were corrected for background activation of the LexA reporter alone.

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The secondary structural features of the 5.5 LRR repeats and approximate locations of LRR mutations that significantly decreased binding between CCR4 and CAF1 and which affected CCR4 enzyme activity are shown. The predicted CCR4-LRR secondary structure is based upon the crystal structures of the prototypical LRR human and porcine RI proteins (28, 41).

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Fig. 3. Schematic representation of the LRR domain of CCR4. The secondary structural features of the 5.5 LRR repeats and approximate locations of LRR mutations that significantly decreased binding between CCR4 and CAF1 and which affected CCR4 enzyme activity are shown. The predicted CCR4-LRR secondary structure is based upon the crystal structures of the prototypical LRR human and porcine RI proteins (28, 41).

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the genome. Each of these CCR4 proteins was expressed at comparable levels (Fig. 4A, right panel; although the abundance of CCR4 and CAF1 in the wild-type lane appears diminished relative to that in the mutants, other analyses indicate that there is no effect of the mutations on CCR4 or CAF1 protein abundance). Coimmunoprecipitation experiments were carried out to compare the binding of native CAF1 to wild-type CCR4 and CCR4-LRR mutants. Whole-cell extracts from wild-type and LRR mutant strains were treated with polyclonal rabbit anti-CAF1 antibody and the resultant immunoprecipitated proteins were subjected to SDS-PAGE and Western blotting using an anti-CAF1, anti-CCR4, and anti-NOT1 antibodies. As anticipated from the two-hybrid assay results, CCR4 was immunoprecipitated from the T360A/R361A LRR mutant strain at levels indistinguishable from that of the wild-type strain (Fig. 4A, left panel), but barely detectable levels of CCR4 were coimmunoprecipitated from the D357A/F358A LRR mutant strain (left panel). NOT1 was coimmunoprecipitated from each of the CCR4-LRR mutant strains and from the wild-type strain at comparable levels, indicating that CAF1 could immunoprecipitate NOT1 even though it failed to bind well with the D357A/F358A CCR4-LRR variant. These results confirm that CAF1 contacts CCR4 through residues D357A/F358A. As indicated above comparable experiments could not be conducted for the CCR4-CAF16 interaction, and in vitro binding assays between CAF16 and CCR4 have not proven successful (data not shown).

In contrast to D357A/F358A, which affected CAF1 two-hybrid interactions specifically, the Q429A/F430A and G432F alterations, which affected the two-hybrid interactions to all ligands that were tested, did not interfere with the physical interaction between CCR4 and CAF1 as analyzed by immunoprecipitation (Fig. 4A, left panel). These results suggest that the two-hybrid results with Q429A/F430A and G432F, and with D386A and Y409A as well (see below), are not indicative of specific defects in contacting CAF1 or, alternatively, the two-hybrid analysis are more sensitive at detecting CCR4-CAF1 interactions than are immunoprecipitations.

We further analyzed the ability of the CCR4-LRR variants to immunoprecipitate CAF1 using CCR4-FLAG versions of a number of CCR4-LRR mutations. The CCR4-FLAG variants were expressed from a GAL1 promoter in a strain deleted for ccr4, and the ability of the CCR4-FLAG proteins to copurify CAF1 was monitored by Western analysis. Expression for each of the CCR4-FLAG variants was comparable with that of wild-type CCR4 (data not shown). As shown in Fig. 4B, only D357A/F358A affected the ability of CAF1 to associate with CCR4.

These data and the preceding immunoprecipitations demonstrate that CAF1 contacts CCR4 primarily through a single site at the α-helix/β-sheet junction of the second LRR repeat.

The LRR Interacts with the C-terminal Exonuclease Domain—The above LRR mutations identified residues important to binding CAF1 within the context of the full-length CCR4 protein. It was possible, however, that other regions of the CCR4 protein helped stabilize the CAF1 interaction and might compensate for LRR mutations that would otherwise affect CAF1 contacts. To assess the importance of the C-terminal exonuclease segment of CCR4 to CAF1 binding, we reassayed a number of LRR mutations for their effects on binding to CAF1 after deletion of the C-terminal 480–837 residue region of CCR4. Two-hybrid analysis of these LexA-CCR4-1–479 variants indicated that in several cases (R383A/V384A, D386A, and G432F) CCR4 contacts to CAF1 were decreased significantly by removal of the C terminus (Table I). In the cases of D386A and G432F, no two-hybrid interactions was observed with B42-CAF1 or any other interactor (data not shown), suggesting that these mutations in the absence of the C terminus had a major effect on the protein. These observations suggest that the exonuclease domain helps stabilize the LRR in its contact to CAF1. One possible way that this would occur would be for the exonuclease domain actually to contact the LRR region.

We therefore examined whether the N terminus of CCR4 could interact with the C terminus of CCR4. Two-hybrid analysis confirmed a physical interaction between the LexA-CCR4-1–479 moiety and the B42-CCR4-490–837 moiety (550 units/mg of β-galactosidase activity compared with less than 10 units/mg for control interactions between the LexA moiety and B42-CCR4-490–837). This interaction was reduced 2-fold by several mutations that were unaffected in their interaction...
Mutagenesis of CCR4-LRR Domain

### Table I

| Mutation         | Relative β-galactosidase activity |
|------------------|-----------------------------------|
|                  | LexA-CCR4 | CAF1-CCR4 |
| Wild-type        | 100       | 100       |
| D357A/F358A      | 5         | 5         |
| T360A/R361A      | 92        | 73        |
| R383A/V384A      | 95        | 32        |
| D386A            | 38        | 0         |
| G432F            | 32        | 0         |
| Y409A            | 45        | 21        |
| Q429A/F430A      | 17        | 23        |
| C426A            | 130       | 88        |
| Y363A            | 42        | 49        |
| S388A/H389A      | 130       | 110       |
| F411A/D412A      | 90        | 94        |

Two-hybrid interaction between LexA-CCR4 variants and B42-CAF1

Two-hybrid assays were conducted as described in Fig. 2. S.E. values were less than 20%. The relative β-galactosidase assay values are given as a percentage of the interaction of wild-type LexA-CCR4-1–837 with B42-CAF1 (2,000 units/mg) or LexA-CCR4-1–479 with B42-CAF1 (4,700 units/mg).

with CAF1 (S388A/H389A, F411A/D412A, and C426A), suggesting that the contacts made by the C terminus were to a separate region of the LRR (data not shown). As expected, the LRR mutations clustered in the β-sheet which affected multiple two-hybrid interactions also affected binding to the C terminus of CCR4, whereas the interactions that specifically affected binding to CAF1 (D357A/F358A) or CAF16 (T360A/R361A) had no effect on LRR interactions with the C terminus (data not shown). The interactions between the separated N- and C-terminal regions could not be confirmed by coimmunoprecipitation, however, suggesting that they may be weak or fragile (data not shown).

The LRR Is Required for CCR4 Deadenylase Activity—The results in the previous section describing an interaction of the LRR and the exonuclease domain of CCR4 suggest also that the LRR may be important for CCR4 deadenylase activity. Expression of the C-terminal exonuclease domain by itself as either a GST-CCR4-495–837 or CCR4-490–837-FLAG fusion resulted in proteins completely inactive using our standard deadenylase assay with RNA substrates containing different poly(A) lengths (data not shown). Although it could be argued that both of these proteins were incompletely folded, comparable homologs to proteins were expressed to comparable levels, and careful quantification of CCR4 protein abundance was done by Western analyses, see Figs. 5 and 4B; data not shown).

Several LRR mutations significantly reduced CCR4 deadenylase activity in vitro (Figs. 5C and 6). For instance, T360A and R361A reduced CCR4 activity by 14-fold (Fig. 6A), R383A/V384A and D386A by 7-fold, and Y363A and Q429A/F430A by 4-fold (Fig. 6). These effects are unlikely to be the result of simply misfolded proteins because these mutations did not alter CCR4 binding to CAF1 (Fig. 4). However, not all LRR mutations severely affected CCR4 deadenylation. For example, the D357A/F358A alterations that specifically interfered with CAF1 binding remained relatively active compared with these other alterations with about 70% of the wild-type protein activity (Fig. 6). As summarized in Fig. 6, all of the mutations that severely affected CCR4 enzymatic activity in vitro clustered to the β-sheet surface of the LRR. In contrast, the mutations prior to or after the β-sheet had much less or no effect on CCR4 in vitro activity. The effect of these LRR mutations on CCR4 in vitro enzymatic activity implicates a wide surface of the β-sheet structure that is required for CCR4 activity.

Because of the effects of several of these LRR alterations on CCR4 in vitro enzymatic activity, we examined the effect of these CCR4-FLAG variants on CCR4 function in vivo. The CCR4-FLAG LRR variants used for the enzyme assays were expressed from a GAL1 promoter, which allowed us to assay their function under glucose growth conditions when their abundance was comparable with a single chromosomal copy of the CCR4 gene (data not shown) as well as under galactose growth conditions when their abundance was about 20-fold higher. As depicted in Table II, deletion of either the CCR4 deadenylation domain or two of its LRR repeats completely abrogated its function in vivo under all conditions analyzed. In contrast, all mutants containing single or double alterations complemented ccr4 for the ability to grow on medium containing glucose and 8 mM caffeine, although the Q429A/F430A mutation displayed weaker growth. These results were nearly identical to what was observed for the complementation ability of the LexA-CCR4 variants as described above. Under galactose growth conditions, however, at the higher temperature of 34 °C, a number of CCR4-LRR-FLAG variants defective in CCR4 enzymatic function, such as T360A/R361A, R383A/V384A, D386A, Q429A, F430A, and G432F, resulted in an inability to grow on 8 mM caffeine-containing plates (Table II). Other mutations outside the β-sheet region which did not affect CCR4 in vitro activity correspondingly complemented ccr4 completely. However, two β-sheet alterations, Y363A and Y409A, which displayed reduced activities, appeared to function normally in vivo, suggesting that other protein factors may be influencing CCR4 function in vivo.

DISCUSSION

LRR Sites of Interaction with Its Protein Ligands—Using systematic mutagenesis of the CCR4-LRR region, the LRR contacts to CAF1 and CAF16 have been defined. CAF1 contacts CCR4 through the specific α-helix/β-strand loop residues Asp-357 and Phe-358. This was verified by both two-hybrid analysis and immunoprecipitations. The results of this study are also highly suggestive that mutation of the β-strand residues Thr-360 and Arg-361 abrogates the physical binding of CAF1 to the LRR, although more direct evidence of the effect of LRR mutations on the physical association of CCR4 with CAF16 is needed. Our data show that a typical small LRR domain, such as that of CCR4, can accommodate multiple protein ligands and stabilize their association through a relatively few strong contacts. It is not clear, however, whether these ligands can bind to a single LRR domain simultaneously. Extrapolation of our findings to proteins containing more extensive LRRs suggests that many separate ligands could, in theory, bind to a single LRR interface.

Site-directed mutagenesis data available for the structural basis of human RI binding to two of its ligands, human angiogenin and bovine RNase A (23, 30), indicate that although several residues well conserved among RNase family proteins may serve as common points of attachment, RI largely employs...
different LRR contacts for binding to distinct ligands. Although the binding interfaces of RNase A and with human RI and porcine RI, and of angiogenin with human RI, are extensive and involve multiple domains of each binding pair, the most energetically important contacts between each of these binding pairs reside in a small C-terminal segment of the LRR domain (23, 30, 41). Replacement of several nonconserved neighboring
residues substantially weakens the affinity of porcine RI for RNase A but has little effect on binding to angiogenin (30). Likewise, CCR4 employs a short N-terminal LRR region to bind to CAF1 and to CAF16. Although we are unable to rule out the possibility of additional CCR4 contact residues within, or outside of, the LRR region, for each of these molecular complexes, relatively restricted interactions appear to provide the major anchors for attachment.

All of the amino acid substitutions that strongly decreased or completely abrogated CCR4-LRR binding to CAF1 and CAF16 were localized to β-strands or the loop connecting the C terminus of the first α-helix with the N terminus of the second β-strand (Fig. 3). Although our scanning mutagenesis of the CCR4-LRR was not exhaustive, we did not identify any α-helix residues that impacted protein ligand binding. The β/α-loop region of CCR4, although not involved in contacting CAF1 or CAF16, did, however, affect the two-hybrid interaction between the LRR and the C terminus of CCR4. Previous studies have shown that any solvent-exposed LRR residue may potentially participate in ligand binding. For instance, LRR residues in the extracellular domain of rat luteinizing hormone/chorionic gonadotropin (CG) receptor which affect binding to human CG include α-helix and β-strand residues as well as loop regions (42). Yet, a majority of previous studies for which LRR mutagenesis data are available have concluded that residues of the LRR β-sheet and α/β-loop regions are those most likely to be involved in ligand binding. For example, the human RI angiogenin contact surface encompasses 25 residues on 13 different repeat units. 24 of these residues lie on β-sheets or β/α-loops; just one is an α-helix residue (23). Similarly, the majority of porcine RI LRR residues that form close contacts with RNase A occur in the loops connecting the C termini of the β-strands with the N termini of the α-helices (41). Residues of the Ran-GTPase-LRR required for Ran binding occur in the β/α-loops (43). The binding specificities of two LRR proteins, polygalacturonase-inhibiting protein and the flax P2 resistance gene, have also been shown to reside in residues of the LRR β-sheet and α/β-loop regions (31, 44). Our CCR4 mutagenesis data support these findings in implicating the LRR β-sheet and α/β-loops as key binding regions.

Several LRR mutations caused a general and significant overall decrease in the interaction of CCR4 to multiple partners: Q429A/F430A, Y409A, D386A, and G432F. Both the Q429A/F430A and the Y409A alterations also resulted in stronger activation of the LexA reporter in transcriptional assays. Because mutation of residues Q429/F430 and Y409 broadly impacts the CCR4 protein both positively and negatively, these alterations, and possibly the others, may affect the overall structure or topology of the LRR, leading to the loss of binding specificity in two-hybrid assays. Effects on the LRR could also influence contacts to the C-terminal activation domains of CCR4 (33), resulting in the higher activation of transcription which was observed. Most importantly, the β-sheet mutations could be affecting CCR4 by affecting contacts to the deadenylase domain. In addition, the β-sheet mutations R353A/V354A, D386A, and G432F displayed significant, if not complete, loss of ability to bind CAF1 when the C-terminal deadenylase domain was removed. These results suggest a critical interaction between the LRR and the exonuclease domain in binding CAF1. Therefore, although we were only able to identify directly two residues critical for binding CAF1 (Asp-357 and Phe-358), other residues throughout the β-sheet may play important roles in contacting CAF1 and/or the exonuclease domain.

Significantly, the two CCR4 mutants for which we were completely unable to obtain protein expression were the double mutants K406A/Y407A and C426A/N427A. Lack of CCR4 expression in these mutants may be attributable to protein instability resulting from structural changes. Residues Lys-406, Tyr-407 and Cys-426, Asn-427 lie proximal to Gln-429, Phe-430, and Tyr-409, which also appear to impact the LRR. It is noteworthy that as single mutations, C426A, N427A, K406A, and Y407A all expressed stable and functional CCR4 proteins. None of these latter variants significantly altered binding to CAF1, CAF16, or CAF17 proteins or resulted in high transcriptional activation, although C426A did affect by 2-fold the interaction with the C terminus of CCR4. These observations suggest that some interstial residues may contribute to a cooperative network of intramolecular bonds that stabilize LRR structure or promote its function, especially when a pair of residues is mutated together. Previous studies support this hypothesis in that residues in the β-sheet structure have been found to be involved in intramolecular LRR hydrogen bonding (28).

The CCR4-LRR Is Critical for Deadenylase Function—The LRR of CCR4 has been shown previously to be absolutely required for CCR4 in vivo function (14) and to be the region through which CCR4 binds CAF1 (10). Because a caf1 deletion results in phenotypes similar to that of deleting cer4, it has been concluded previously that the LRR is essential solely as a consequence of its binding CAF1. We report here, however, that the LRR plays an additional role in being absolutely required for CCR4 deadenylase activity. The CCR4 exonuclease domain by itself was nonfunctional in vitro, and deletion of the LRR repeats completely inactivated CCR4 in vitro. Importantly, a number of LRR mutations across the β-sheet surface significantly reduced CCR4 activity. That so many different alterations in the LRR affected CCR4 activity in vitro implies that the LRR makes multiple critical contacts essential for deadenylation.

The CCR4-LRR could be important to deacylation by either contributing to the stabilization and proper formation of the deadenylase domain or in aiding the deadenylase domain in binding its RNA substrate. The U2A′ LRR-containing protein utilizes its LRR to contribute toward stabilizing the binding of U2B′ to its cognate RNA (25). Although this function acts in trans for U2A′, the CCR4-LRR could function similarly to help stabilize the exonuclease domain in its binding to its RNA substrates. Our observation that the CCR4-LRR can make two-hybrid contact to the CCR4 deadenylase domain supports this hypothesis. Alternatively, the CCR4-LRR could make direct contact to the RNA as was found for the LRR of the TAP mRNA export factor (22). In the case of TAP, the LRR mutations that specifically blocked RNA binding were located in the loop region between the β-sheet and the α-helix. However, for CCR4 the LRR mutations that reduced its function clustered exclusively to the β-sheet surface. It may be possible that the CCR4-LRR may be both contacting the exonuclease domain and binding to the RNA. We believe it is less likely that the LRR is directly involved in catalysis in that in vivo the LRR mutations are not defective under all growth conditions.

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Addendum—A systematic mutagenesis of the β-sheet residues in the LRR region of the CG receptor has now been conducted (45). In this study, CG hormone recognition specificity was changed to that of thyrotropin or to follicle-stimulating hormone receptor by altering eight or two residues, respectively, of the CG LRR. These data confirm the importance of discrete regions of the β-sheet of the LRR in making its protein contacts. Moreover, specific intramolecular LRR electrostatic interactions were suggested for these hormone receptors, an observation that implies that Arg-361 and Asp-386 of the CCR4-LRR may also be involved in such an interaction.
Systematic Mutagenesis of the Leucine-rich Repeat (LRR) Domain of CCR4 Reveals Specific Sites for Binding to CAF1 and a Separate Critical Role for the LRR in CCR4 Deadenylase Activity
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