Identification of Residues in the L1 Region of the RecA Protein Which Are Important to Recombination or Coprotease Activities*

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Using a combinatorial cassette mutagenesis procedure we have introduced a large number of single and multiple amino acid substitutions into an area of the RecA protein defined by residues 152-159. This sequence overlaps the disordered loop 1 region (L1) in the RecA crystal structure which has been hypothesized to be involved in DNA binding. Assays for recombinational DNA repair and LexA coprotease activities identify Glu154 as the only one of these 8 residues which is critical for RecA function. Several other mutations observed at nearby residues support the identity of Glu154 as the most important of the 14 residues in the area defined by Pro152 to Met158. In addition, Glu157 and Glu158 appear to be hot spots for the occurrence of mutation-induced constitutive coprotease activity.

The RecA protein from Escherichia coli is a multifunctional enzyme that plays two distinct catalytic roles related to cell survival following DNA damage. First, RecA catalyzes a postreplication strand exchange activity between homologous DNA molecules so that information from the undamaged DNA is used to restore information on the damaged homologous partner (for review see Refs. 1-4). Second, in response to DNA damage RecA becomes activated for a coprotease function in which it facilitates the autoproteolysis of the cellular LexA repressor (5, 6). This event increases the expression of a number of genes including recA, collectively referred to as the SOS genes, which are involved in cell survival following DNA damage (7, 8). To carry out either of these activities the RecA protein must bind both ATP and DNA to form an activated nucleoprotein filament. Even though a number of recA mutants show coincident decreases in both recombination and coprotease activities, there is both genetic and biochemical evidence showing that these two activities are separable. A number of recA mutants have been shown to be coprotease-proficient but compromised for recombination activity (9, 10). In addition, several mutations result in RecA proteins that display a constitutive coprotease activity (catalysis of LexA cleavage in the absence of DNA damage) yet have varying effects on the recombination activity (11, 12, this study). We have now designed a series of coprotease-proficient but compromised for recombination activity (9, 10).

To investigate the structural requirements for the many activities catalyzed by RecA we have initiated mutagenesis studies within targeted regions of the protein (13-15). The availability of the x-ray crystal structure of RecA (16, 17) provides an opportunity to address detailed questions regarding the functional and/or structural roles of specific amino acid residues within these targeted regions. The structure of RecA was solved in the absence of bound DNA, and Story et al. (16) have suggested that two disordered regions, L1 and L2, are involved in the interaction of the RecA oligomer with DNA. We have introduced a large number of single and multiple amino acid substitutions into a stretch of 8 residues (152-159) that flank and are contained within region L1. Assays for both the DNA repair and LexA coprotease activities show that RecA function is most sensitive to mutation at Glu154. The DNA repair activity shows a moderate sensitivity to mutation at Glu157, whereas the other six positions tolerate high levels of substitution. Twenty-nine of the 149 unique mutants in this study display constitutive coprotease activity yet have widely varying effects on DNA repair activity. Our results are discussed in terms of possible roles for this region in the catalysis of LexA autodigestion and DNA repair.

EXPERIMENTAL PROCEDURES

Materials—All media (LB broth, LB agar, 2 x YT) were prepared as described (18) and were supplemented with 100 µg/ml ampicillin when appropriate. MacConkey-lactose plates were prepared according to the manufacturer (Difco) and contained 0.5% lactose and 100 µg/ml ampicillin. Stock solutions (4 mg/ml) of o-nitrophenyl β-D-galactopyranoside (ONPG; Sigma) were made in Z-buffer (19). Protein concentrations in cell extract supernatants were determined using the Bio-Rad protein assay kit. Mitomycin C was from Sigma or Boehringer Mannheim. Restriction enzymes, polynucleotide kinase, T4 DNA ligase, and Klenow DNA polymerase I large fragment were from New England Biolabs. Modified TT DNA polymerase (Sequenase version 2) was from U. S. Biochemical Corp. All buffers were as recommended by the manufacturer. Turker containing polynucleotide antibodies against RecA (rabbit anti-RecA) was prepared commercially (Ea. Inc., Lexington, MA) using gel-purified RecA protein as antigen (13).

Strains and Plasmids—Strain X90 (20) was used for general purposes, e.g. plasmid construction and propagation and as a recA control strain. Strain DE1663 was used for in vivo assays of the DNA repair and coprotease activities of all plasmid-borne recA mutanta. DE1663 was constructed by mating strain DE1663, a Δ(recA-srZR) 3061Tn10 Δ(lac-argF) U169 sulA211 mndB1TsB (a cl ind-1 recA84/p: lac ZY) derivative of AB1157, with strain DE1781 which carries an F' lac operon. Both DE1663 and DE1781 were generous gifts from Don Ennis (NIH).

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1. J. T. Konola, H. G. Nastri, and K. L. Knight, unpublished data.

2. The abbreviations used are: L1 and L2, loop 1 and loop 2; ONPG, o-nitrophenyl β-D-galactopyranoside.

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This plasmid is a derivative of pTRecA103 (13). Unique SalI and BssHII restriction sites were introduced at positions denoted for the measurement of RecA-mediated LexA cleavage by determination of β-galactosidase activity in extracts of cultures carrying the recA mutants. Cultures were grown overnight at 37 °C, diluted 1/100 in LB-ampicillin media, and grown for 2 h at 37 °C. The cultures of each culture (1.5 ml) mitomycin C was added to a final concentration of 0.5 µg/ml, and all samples, both (+) and (−) mitomycin C, were grown for an additional 40 min. Cultures were then chilled on ice for 20 min, centrifuged for 5 min, washed with 1 ml of 10 mM NaCl, and resuspended in 1 ml of Z buffer (19). Cells were sonicated on ice for 30 s, centrifuged for 20 min, and the supernatant was stored at 4 °C. β-Galactosidase activity in these supernatants was measured using ONPG as described (19). Units of β-galactosidase are defined as 10⁻³ mol of ONPG hydrolyzed/min, and activity is expressed as units/µg protein.

Cellular Level of Mutant RecA Proteins—Western blot analysis was performed as described previously (13) for each recA mutant. For this analysis cultures were grown in the absence of isopropyl-1-thio-β-D-galactopyranoside. None of the mutants described in this study showed a steady-state level of RecA protein which was significantly different from wild type RecA data not shown).

DNA Sequencing—Amino acid substitutions were determined by DNA sequence analysis of the plasmid-borne mutant recA genes. Sequencing was performed using modified T7 DNA polymerase (Sequenase version 2) on either double-stranded template (Magic Mini-prep DNA purification system; Promega) or single-stranded template that was isolated following infection of cultures carrying the recA region of a plasmid with an M13-digested RecA plasmid, as described previously (26). We determined the sequence of the cassette insert as well as several bases flanking the SalI and BssHII restriction sites.

RESULTS

463 transformants were screened for DNA repair and LexA coprotease activity. DNA sequence analysis showed that 254 mutants contain one of the following: 1) silent mutation(s); 2) an insertion or deletion; 3) nonsense codon(s); or 4) the wild type recA sequence. Data for the remaining 209 mutants (149 unique) are presented below. Previous studies suggest that cell survival following DNA damage by UV or mitomycin C is more a measure of the recombination proficiency of RecA than its ability to induce the SOS response (27, 28). Two results, in particular, lend strong support to this claim: 1) transformation of pTRecA322 into a strain deleted for recA in which the SOS system is irreversibly repressed (ΔrecA lexA3) allows survival following exposure to UV or mitomycin C at approximately 80% of the level seen in either a ΔrecA lexA strain carrying the same plasmid or a recA+ lexA strain2; and 2) a strain deleted for recA in which the SOS system is expressed constitutively (ΔrecA lexA del) shows survival at no more 0.5% the level of a recA+ lexA strain3 (29). Therefore, using survival following DNA damage as a measure

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of the recombinational proficiency of RecA we separated all mutants into three general phenotypic categories defined as follows. recA+ showed growth in the presence of both 0.3 and 0.6 µg/ml mitomycin C and survival of UV irradiation (fractional survival at 30 s ≤ 0.95) similar to positive control cells (DE1663’/pTR3RecA322 or X90). recA- showed no survival following exposure to mitomycin C or UV (fractional survival at 30 s ≤ 0.05) greater than negative control cells (DE1663’/pZ100). recA+ showed intermediate levels of survival.

Preliminary screens for LexA coprotease activity were performed using MacConkey-lactose plates (±mitomycin C). More quantitative ONPG assays were used to separate all mutants into three categories: coprt+ (constitutive activity), coprt- (activity induced following mitomycin C-dependent DNA damage), and copr-. These categories are defined as follows. Coprt+ represents noninduced β-galactosidase activity at least 1.5-fold higher than wild type RecA. Coprt- represents noninduced activity greater than negative control cells (DE1663’/pZ150) followed by some degree of mitomycin C-dependent induction. Coprt- represents noninduced and induced levels of activity similar to negative control cells. Copr- were mutated into three categories based on the specific activity of β-galactosidase in the absence of mitomycin C: strong, >20 units/µg; moderate, <20 and ≥10; weak, <10 and ≥5.8.

Lys152—Position 152 tolerates a high level of mutation with no adverse effects on either the DNA repair or coprotease activities. The Lys → Ala recA'/copr+ mutant indicates that no specific side chain information is required at this position for either activity (Table I). Several other substitutions allow full activity and are limited to positively charged (Arg and His) or uncharged, polar residues (Gln, Asn, Ser, and Thr).

A significant inhibition of the DNA repair activity results from mutation to a negatively charged (Glu) or hydrophobic (Ile) side chain (Table II, N-82 and N-83). In contrast, although the Glu mutation decreases coprotease activity this function is unaffected by the Ile substitution. The deleterious effects of both substitutions are suppressed by a secondary change to Lys at nearby positions (see below; Tables I and V, mutants N-43 and N-45). No single substitution at position 152 was found which completely inhibited RecA functions.

Ala153—Position 153, like 152, accommodates a number of substitutions that have no effect on either RecA activity. Both polar (Ser, Thr, and Gln) and nonpolar (Leu and Val) residues are allowed. Substitution by either Pro or Gly is also allowed, indicating that no particular constraints exist regarding the positioning of the polypeptide backbone at this location.

Like position 152, introduction of a negatively charged Glu side chain results in a decrease in DNA repair activity (Table II, N-84). However, this mutation increases coprotease activity to a moderately constitutive level (see Table IV). No single substitution was found at this position which completely inhibited RecA activity.

Glu154—This is the only one of the 8 targeted residues for which the wild type side chain appears to be critical for the maintenance of full RecA activity. Although we picked up only four unique single mutations, they indicate that rather strict chemical and steric constraints are in effect at this position. A Glu154 → Asp mutation results in an appreciable decrease of both activities (Table II, N-85), whereas substitution with an isosteric Gln residue completely inactivates RecA functions (Table III, N-121). These two mutants suggest that both the presence and precise positioning of a negative charge at position 154 are critical to RecA function. Interestingly, defects in the DNA repair versus coprotease activities of the Glu154 → Asp mutant are differentially suppressed by second site mutations (see Table V, N-85, N-95, N-95; see below).

Mutation of Glu154 to either Lys or Arg completely inactivates RecA (Table III, N-122 and N-123). Other mutations at this position that probably account for a recA+ phenotype (Gly, Ala, Tyr, and Val) have been inferred from multiple substitution mutants (see below).

Ile155—Position 155 tolerates a number of substitutions of varying chemical character and size with little or no adverse effect on either RecA activity. Substitution with other nonpolar residues of varying size (Ala, Val, and Leu), polar residues Cys and His, and the aromatic Phe and Tyr residues have no detectable effect on DNA repair or coprotease activity (Table I). However, although an Ile155 → Met mutation has no effect on the DNA repair function, it has a dramatic effect on coprotease activity, resulting in a strong copr+ mutant (Table IV, N-19). The fact that this is the only one of 8 single mutations which has an exclusive effect on the coprotease function suggests that there are specific chemical and/or steric requirements at this position which differentiate between the DNA repair and coprotease capabilities of RecA.

Substitution of Ile155 with Asn has a slight inhibitory effect on both activities (Table II, N-86). Although we found no single substitution that completely inactivates RecA function, mutation to Thr appears to correlate with complete inhibition of both activities. A double mutant carrying Ile155 → Thr and Lys156 → Thr (Table III, N-128) scores as recA+/copr+ even though the latter mutation by itself has no effect on RecA function (Table I, N-6).

Gln156—A large number of mutations are tolerated at residue 156, indicating that virtually no constraints, chemical or steric, exist at this position (Table I). The fact that Gly allows full activity indicates that there is no essential information regarding side chain identity or polypeptide backbone conformation.

In addition to Gly and a conservative Asp mutation other single substitutions that have no effect on the DNA repair activity include hydrophobic (Ala, Val, and Ile) and positively charged (Arg and Lys) residues (Table I). Mutation to Gln and Leu occurs in multiple substitution mutants that also score as recA+.

Similar to residue 155 there appears to be very specific requirements that allow an exclusive effect on coprotease activity at position 156. Mutation to either Lys or Arg has no effect on DNA repair activity, yet only the Lys substitution results in a strong copr+ mutant (Table IV, N-29). Lys is the only one of seven recA+ single mutants which shows such an effect.

No single mutation was observed to inhibit RecA activities completely. For each of the four recA+/copr+ multiple mutants carrying a change at position 156 the phenotype can be attributed to one of the other mutations (Table III, N-142, N-143, N-147, N-148).

Glu157—In addition to Glu154 this is the only other one of the 8 targeted residues which shows some sensitivity to mutation. Only two single mutants were observed, Cys and Asp, both of which result in decreased DNA repair activity (Table II, N-87 and N-88). Interestingly, both of these mutations resulted in constitutive coprotease activity (see below). The Gly157 → Asp mutant corresponds to recA1602, and our results match the previous characterization of this mutant as having partial DNA repair activity and moderate constitutive coprotease activity (11, 12).

Unlike position 154 several substitutions at position 157 result in little or no inhibition of DNA repair activity. For example, although Gly157 → Ser was not obtained as a single mutant, the three multiple mutants with this change score either as recA+ or show very high partial activity (Table I, N-22 and N-77; Table II, N-106). Changes to either Trp or Arg are observed in two triple mutants that retain a significant amount
of DNA repair activity (Table II, N-117 and N-118). Also, substitution to Ala is seen in four multiple mutants, one of which retains a very high partial activity (Table II, N-113), the others maintaining moderate levels of activity (Table II, N-97, N-104, and N-105). Although we cannot say with certainty that single changes at position 157 to Ala, Trp, or Arg would not by themselves decrease DNA repair activity, these results suggest that position 157 tolerates at least a moderate level of mutation.

In contrast to the DNA repair activity, mutations at position 157 had a significant effect on coprotease activity, frequently resulting in coprt' mutants. Of the 20 mutants in this study which contain a substitution at Gly157, 10 are coprt' (Table IV), a result suggesting that this position is particularly sensitive to mutation-induced constitutive coprotease activity (see below).

The two multiple mutants that contain Gly157 → Cys provide another example of suppressors that differentially alter defects in the DNA repair versus coprotease activities of the primary mutant (Table V, N-87, N-70, N-120; see below).

Glu158—Position 158 supports a high level of mutation, and there appear to be very few constraints at this position regarding DNA repair activity. Single mutants that score as recA* include positively charged (Lys), uncharged polar (Gln and...
Sequences of the 39 recA" mutants observed in this study are shown. No recA" mutants were observed with \( \geq \) four substitutions. Assays for both recombinational DNA repair and LexA coprotease activities are described under "Experimental Procedures." Assays were repeated at least three times for each mutant. Asterisks indicate copr" mutants (see Table IV). Numbers in parentheses refer to the number of mutants with the indicated substitutions which arose independently, and for these mutants the scores for UV survival were averaged and include the standard error of the mean. Standard errors are not shown for all \( \beta \)-galactosidase measurements but approximate those shown for the positive and negative controls.

### Table II

| recA" mutants | 150 | 155 | 160 | 164 | mito C | UV survival | \( \beta \)-gal |
|---------------|-----|-----|-----|-----|--------|-------------|--------------|
| N-82          | 0   | 0   | 0   | 0   | 1/0    | 0.08        | 2.3          |
| -83           | 0   | 0   | 0   | 0   | 2/0    | 0.55        | 4.7          |
| *-84 (2)      | 0   | 0   | 0   | 0   | 1/0    | 0.59±0.17   | 8.6          |
| -85 (2)       | 0   | 0   | 0   | 0   | 2.5/1.5| 0.23±0.18   | 2.3          |
| -86 (5)       | 0   | 0   | 0   | 0   | 3.4/2.1| 0.92±0.03   | 2.1          |
| *-87          | 0   | 0   | 0   | 0   | 3.5/0  | 0.46        | 10.5         |
| *-88          | 0   | 0   | 0   | 0   | 1.5/0  | 0.11        | 10.5         |
| -89           | 0   | 0   | 0   | 0   | 4/3    | 0.48        | 3.1          |
| *-90 (2)      | 0   | 0   | 0   | 0   | 4/1    | 0.60±0.29   | 4.0          |
| *-91          | 0   | 0   | 0   | 0   | 4/4    | 0.84        | 2.9          |
| -92           | 0   | 0   | 0   | 0   | 1/0    | 0.08        | 4.0          |
| *-93          | 0   | 0   | 0   | 0   | 4/4    | 0.25        | 24.1         |
| -94           | 0   | 0   | 0   | 0   | 4/4    | 0.85        | 3.3          |
| -95           | 0   | 0   | 0   | 0   | 2/1    | 0.35        | 4.1          |
| *-96          | 0   | 0   | 0   | 0   | 4/2    | 0.75        | 27.9         |
| *-97          | 0   | 0   | 0   | 0   | 3.5/0  | 0.74        | 10.7         |
| *-98          | 0   | 0   | 0   | 0   | 0/0    | 0.11        | 11.4         |
| *-99          | 0   | 0   | 0   | 0   | 0/0    | 0.08        | 13.2         |
| -100          | 0   | 0   | 0   | 0   | 4/4    | 0.42        | 4.5          |
| -101          | 0   | 0   | 0   | 0   | 4/4    | 0.82        | 3.7          |
| -102          | 0   | 0   | 0   | 0   | 4/4    | 0.93        | 3.6          |
| -103          | 0   | 0   | 0   | 0   | 1/0    | 0.21        | 3.1          |
| -104          | 0   | 0   | 0   | 0   | 4/4    | 0.59        | 3.1          |
| -105          | 0   | 0   | 0   | 0   | 2/2    | 0.43        | 1.6          |
| *-106         | 0   | 0   | 0   | 0   | 4/4    | 0.93        | 12.3         |
| *-107         | 0   | 0   | 0   | 0   | 2/0    | 0.09        | 6.1          |
| -108          | 0   | 0   | 0   | 0   | 4/3    | 0.89        | 2.1          |
| *-109         | 0   | 0   | 0   | 0   | 2/1    | 0.27        | 29.4         |
| *-110         | 0   | 0   | 0   | 0   | 1/0    | 0.10        | 9.7          |
| -111          | 0   | 0   | 0   | 0   | 2/2    | 0.10        | 2.3          |
| *-112         | 0   | 0   | 0   | 0   | 1/0    | 0.47        | 11.0         |
| -113          | 0   | 0   | 0   | 0   | 4/4    | 0.80        | 5.0          |
| *-114         | 0   | 0   | 0   | 0   | 0/0    | 0.10        | 8.4          |
| -115          | 0   | 0   | 0   | 0   | 4/4    | 0.93        | 2.8          |
| -116          | 0   | 0   | 0   | 0   | 2/0    | 0.04        | 3.8          |
| -117          | 0   | 0   | 0   | 0   | 4/4    | 0.52        | 4.7          |
| -118          | 0   | 0   | 0   | 0   | 4/4    | 0.87        | 2.0          |
| *-119         | 0   | 0   | 0   | 0   | 0/0    | 0.14        | 8.6          |
| -120          | 0   | 0   | 0   | 0   | 4/4    | 0.71        | 5.4          |

| pTRRecA322    | 4/4 | 1.00 | 3.4±0.5 | 8.6±0.7 |
| pZ150         | 0/0 | 0   | 1.6±0.2 | 1.6±0.2 |

Asn, and hydrophobic (Leu) residues (Table I). A number of other mutations at position 158, including Arg, Ser, Ala, Val, Ile and Asp, occur in multiple substitution mutants which score as recA" (Table I). Even the large, aromatic residues Tyr and Phe permit a moderate to high level of repair activity, although these changes occur in multiple mutants (Table II, N-100 and N-115). No single mutation at position 158 resulted in a recA" phenotype.

Many of the mutants with changes at position 158 which had no effect on DNA repair showed constitutive coprotease activity. The frequency of copr" mutants with changes at this position and the varying chemical nature of the substitutions...
resulting in this phenotype suggest that position 158, like 157, is sensitive to mutation-induced constitutive coprotease activity (see below). Our mutant N-30 (Glu158→Gly) corresponds to recA1219, which has also been characterized previously as activities of position 158 mutants (Table V, see below). The only other nontargeted mutation that can be correlated with a recA' coprt' phenotype is Gly158→Ser (Table III, N-133). This substitution occurs in a double mutant along with Ile155→Val, a mutation that by itself has no effect on RecA function (Table I, N-26). This result suggests that the flexibility of the polypeptide chain at position 165 is also important to both RecA activities.

An Asp151→Asn substitution allows full RecA function (Table I, N-37), whereas an Asp151→Val change still allows a moderate level of DNA repair activity and wild type-like coprotease activity (Table II, N-89). This result indicates that not only is a formal negative charge not essential, but the general steric and chemical constraints are somewhat lax at position 161.

A Ser162→Phe recA' coprt' mutant suggests that this position would likely support a high level of mutation (Table I, N-38).

Substitution of His163 with Leu results in a mutant RecA with a moderate level of DNA repair activity and no decrease in coprotease activity (Table II, N-90) indicating that the wild type side chain at this position is not essential for RecA function.

Finally, a Pro161→Ser mutation in the recA' coprt' double mutant (Table I, N-40) suggests that the rotational constraints imposed on the polypeptide backbone by the wild type side chain at this position are not critical to RecA function.

**Multiple Substitution Mutants**—For the most part the DNA repair phenotype of multiple mutants can be understood in terms of the corresponding single mutations. Several mutants, however, proved to be exceptions to this general trend. Based on the repair phenotypes of two recA' single mutants (Lys152→Gln and Ala153→Leu; Table I, N-3 and N-12) one might predict that the corresponding double mutant would be recA'. However, this double mutant (Table II, N-92) shows a repair activity only marginally above the negative control. Likewise, the recA' double mutant N-126 (Table III) carries two substitutions (Lys152→Asn and Ala153→Val), by themselves score as recA' (Table I, N-7 and N-11). These results suggest some deleterious interaction between the side chains at positions 152 and 153 in the two double mutants which does not occur when only one of these positions carries a substitution.

A similar observation was made regarding the DNA repair phenotype of one particular triple mutant (N-116, Table II) which carries three conservative changes, Glu155→Asp, Glu158→Asp, and Ile159→Leu. Based on the occurrence of these

**Table III: recA' mutants**

Sequences of the 29 recA' mutants observed in this study are shown. Phenotypes were scored as described under "Experimental Procedures," and no data are presented because all mutants showed no DNA repair or coprotease activity greater than the negative control. Numbers in parentheses refer to the number of mutants with the indicated substitution(s) which arose independently.

| mutant | T | P | K | A | E | I | G | E | I | G | D | S | H | M | G |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N-121 (3) | . | . | Q | . | . | . | . | . | . | . | . | . | . | . | . |
| -122 (3) | . | . | K | . | . | . | . | . | . | . | . | . | . | . | . |
| -123 | . | . | R | . | . | . | . | . | . | . | . | . | . | . | . |
| -124 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| -125 (A147→V) | V | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| -126 | N | V | . | . | . | . | . | . | . | . | . | . | . | . | . |
| -127 | N | G | . | . | . | . | . | . | . | . | . | . | . | . | . |
| -128 | T | T | . | . | . | . | . | . | . | . | . | . | . | . | . |
| -129 | T | Q | . | . | . | . | . | . | . | . | . | . | . | . | . |
| -130 | N | N | G | . | . | . | . | . | . | . | . | . | . | . | . |
| -131 | V | V | . | . | . | . | . | . | . | . | . | . | . | . | . |
| -132 | Q | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| -133 | V | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| -134 | A | A | . | . | . | . | . | . | . | . | . | . | . | . | . |
| -135 | Y | S | M | . | . | . | . | . | . | . | . | . | . | . | . |
| -136 | T | D | . | (F166→A) | . | . | . | . | . | . | . | . | . | . | . |
| -137 | Q | V | . | . | . | . | . | . | . | . | . | . | . | . | . |
| -138 | I | D | L | . | . | . | . | . | . | . | . | . | . | . | . |
| -139 | I | V | . | . | . | . | . | . | . | . | . | . | . | . | . |
| -140 | P | D | L | . | . | . | . | . | . | . | . | . | . | . | . |
| -141 | V | A | V | . | . | . | . | . | . | . | . | . | . | . | . |
| -142 | H | V | H | . | . | . | . | . | . | . | . | . | . | . | . |
| -143 | V | V | L | . | . | . | . | . | . | . | . | . | . | . | . |
| -144 | Q | L | R | . | . | . | . | . | . | . | . | . | . | . | . |
| -145 | A | T | K | M | . | . | . | . | . | . | . | . | . | . | . |
| -146 | K | C | K | K | . | . | . | . | . | . | . | . | . | . | . |
| -147 | A | E | K | M | V | . | . | . | . | . | . | . | . | . | . |
| -148 | N | T | G | V | . | . | . | . | . | . | . | . | . | . | . |
| -149 | (T167→A) | . | . | . | . | . | . | . | . | . | . | . | . | . | . |

Thr appears to be a deleterious mutation as the double mutant Glu155→Val/Ile159→Thr has low partial activities (Table II, N-138), whereas the Gly158→Val change by itself has no effect on RecA functions (Table I, N-24). Although we did not pick up as many substitutions at position 159 as for other residues in this study, we did not find any that correlate specifically with a recA' or coprt' phenotype.

**Mutations at Nontargeted Residues**—Although our procedure was designed to mutate two sets of four contiguous residues (152–155 and 156–159) we picked up several fortuitous mutations at nontargeted positions.

A Gly156→Ala substitution completely inactivates both RecA functions (Table III, N-124), suggesting an important role for the rotational flexibility of the polypeptide backbone at this position. However, we observed a double mutant with a substitution at position 160 which maintains a very low level of DNA repair activity (Table II, N-107, Gly158→Gly/Gly156→Ser). In this case the backbone conformational flexibility lost as a result of the Gly156→Ser mutation may be partially compensated for by the substitution of Gly for Glu at position 168. Interestingly, this double mutant also displays a moderate constitutive coprotease activity that is further inducible by mitomycin C (Table IV).

The two double mutants which do not occur when the rotational flexibility of the polypeptide backbone at position 165 is also important to both RecA activities.
Mutagenesis of RecA Region L1

TABLE IV

Coprt' mutants

Amino acid substitutions, LexA cleavage, and DNA repair activities are shown for the 29 coprt' mutants observed in this study. The repair phenotype is indicated by fractional survival following exposure to UV for 30 s. β-Galactosidase activity is a direct measure of the LexA coprotease activity and was determined as described under “Experimental Procedures.” β-Galactosidase and UV survival assays were repeated at least three times for each mutant. Standard errors for all β-galactosidase assays are not shown but approximate those shown for the positive and negative controls.

| Table IV |

| 150 | 155 | 160 | 164 | β-gal | repair |
|------|------|------|------|-------|--------|
| mutant | T | P | K | A | E | I | E | G | E | I | G | D | S | H | M | (+)mito c | (+)mito c | phenotype |
| N-19 (3) | M | | | | | | | | | | | | | | | 28.3 | 22.6 | 1.00 |
| N-29 (2) | K | | | | | | | | | | | | | | | 22.4 | 25.7 | 1.00 |
| N-30 (2) | K | | | | | | | | | | | | | | | 25.2 | 24.8 | 1.00 |
| N-31 | | | | | | | | | | | | | | | | 6.5 | 8.5 | 1.00 |
| N-33 (2) | N | | | | | | | | | | | | | | | 12.7 | 14.9 | 1.00 |
| N-43 | E | K | | | | | | | | | | | | | | | 6.7 | 17.4 | 1.00 |
| N-47 | R | V | | | | | | | | | | | | | | | 8.8 | 12.8 | 1.00 |
| N-60 | Q | A | | | | | | | | | | | | | | | 30.4 | 28.9 | 1.00 |
| N-61 | L | R | | | | | | | | | | | | | | | 26.2 | 26.8 | 1.00 |
| N-66 | L | Q | | | | | | | | | | | | | | | 24.4 | 26.9 | 1.00 |
| N-70 | C | K | | | | | | | | | | | | | | | 20.6 | 16.6 | 1.00 |
| N-71 | S | M | | | | | | | | | | | | | | | 28.4 | 30.1 | 1.00 |
| N-73 | A | M | | | | | | | | | | | | | | | 28.2 | 25.3 | 1.00 |
| N-77 | Q | S | M | | | | | | | | | | | | | | | 14.3 | 21.1 | 1.00 |
| N-84 (2) | E | | | | | | | | | | | | | | | 8.6 | 17.6 | 0.59 |
| N-87 | | | | | | | | | | | | | | | | 10.5 | 15.4 | 0.46 |
| N-88 | | | | | | | | | | | | | | | | 10.6 | 16.5 | 0.11 |
| N-93 | V | M | | | | | | | | | | | | | | | 24.1 | 23.5 | 0.25 |
| N-96 | M | I | | | | | | | | | | | | | | | 27.9 | 20.2 | 0.75 |
| N-97 | G | A | | | | | | | | | | | | | | | 10.7 | 15.8 | 0.74 |
| N-98 | V | D | | | | | | | | | | | | | | | 11.4 | 15.5 | 0.11 |
| N-106 | | | | | | | | | | | | | | | | 13.2 | 13.3 | 0.08 |
| N-107 | G | S | | | | | | | | | | | | | | | 6.1 | 12.3 | 0.09 |
| N-109 | R | V | R | | | | | | | | | | | | | | | 29.4 | 26.2 | 0.27 |
| N-110 | R | E | L | | | | | | | | | | | | | | | 9.7 | 12.6 | 0.10 |
| N-112 | R | D | | | | | | | | | | | | | | | 11.0 | 15.5 | 0.47 |
| N-114 | | | | | | | | | | | | | | | | 8.4 | 9.4 | 0.10 |
| N-119 | V | K | M | | | | | | | | | | | | | | | 8.4 | 10.2 | 0.14 |

pTRecA322

| pZ150 |

| 3.4±0.5 | 8.6±0.7 | 1.00 |
| 1.6±0.2 | 1.6±0.2 | 0 |

substitutions in other recA* and recA+ mutants (see Table I, N-28, N-56, N-58, N-69, N-72, and Table II, N-100, N-101, N-102, N-104), one might predict only a minimal effect on RecA function. However, N-116 shows a very low DNA repair activity. The coprotease activity is unaffected in this triple mutant.

A different result that supports our observation of an unexpectedly high tolerance for mutation at most positions in this region occurs in several triple mutants that carry combinations of hydrophobic substitutions. These include the following mutants with the changes indicated at positions 156, 158, and 159, respectively: N-78 (Val, Leu, and Val), N-79 (Val, Leu, and Leu), N-81 (Gly, Val, and Val) and N-115 (Val, Phe, and Val). Whereas the wild type RecA sequence in this region contains two negative charges in a stretch of 5 residues ("5511e-Glu-Gly-Glu-Ile"555), these four mutants create a run of hydrophobic residues which, despite the dramatic chemical and steric differences from wild type, support wild type-like DNA repair and coprotease functions.

The results in the sections above regarding the effects of mutations on DNA repair activity are summarized in Fig. 2. Coprt' Mutants—Of the 149 unique recA mutants characterized in this study we found 29 that were coprt'. The occurrence of coprt' mutants as well as the level of constitutive activity do not necessarily correlate with the DNA repair phenotype of a given mutant.

Fourteen of the 29 coprt' mutants are recA*, five of which carry single amino acid substitutions. Mutants N-19 (Ile155 → Met), N-29 (Glu156 → Lys), and N-30 (Glu156 → Lys) are examples of strong coprt' mutants, whereas mutants N-31 (Glu156 → Glu) and N-33 (Glu156 → Asn) are examples of weak and moderate coprt' mutants, respectively (Table IV). The remaining 15 coprt' mutants show some decrease in DNA repair activity, but this varies widely from nearly recA* to nearly recA+ (Table IV). Three of these mutants carry single substitutions, one being a weak coprt' mutant (N-84, Ala153 → Glu) and the others showing moderate constitutive activity (N-87, Gly157 → Cys and N-88, Gly157 → Asp).

No single substitution at positions 152, 154, 159 or any of the nontargeted residues resulted in a coprt' mutant. Of the nine single mutants obtained at Lys152 (Tables I and II) all showed inducible coprotease activity. At Gly157 all four of the single mutants have coprt phenotypes that correspond to the DNA repair phenotypes, three score as recA+/coprt' (Table III, N-121, N-122, and N-123), and the other is partially functional for both activities (Table II, N-85). At position 159 all three single mutants score as recA+/coprt' (Table I, N-34, N-35, and N-36).
TABLE V
Suppressor mutants

Amino acid substitutions, LexA cleavage, and DNA repair activities are shown for six sets of primary mutants and corresponding suppressor mutants. Second site suppressors were found for eight single mutants having alterations in DNA repair, LexA coprotease, or both activities. Assays are described under "Experimental Procedures." Numbers in parentheses refer to the number of mutants with the indicated substitution which arose independently.

| N-30 (2) | N-83 | N-45 | N-82 | N-43 | N-19 (3) | N-85 (2) | N-95 | N-55 | N-87 | N-70 | N-120 | N-31 | N-63 | N-74 | N-76 | N-105 | N-117 | N-33 (2) | N-53 | pTRecA322 | pZ150 |
|----------|------|------|------|------|--------|--------|------|------|------|------|-------|------|------|------|------|-------|-------|-------|------|--------|------|
| mutant  | T | P | K | A | E | I | E | G | E | G | D | S | H | M | β-gal | repair |
|---------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|-------|---------|
|           |    |    |    |    |    |    |    |    |    |    |    |    |    |        |         |
| N-30 (2) | . | . | . | . | . | . | . | . | . | . | . | K | . | . | . | . | . | . | . | . | 25.2 | 24.8 | 1.00 |
| N-83 | . | I | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 4.7 | 9.4 | 0.55 |
| N-45 | . | I | . | . | . | . | . | . | . | . | . | . | K | . | . | . | . | . | . | 3.7 | 9.1 | 1.00 |
| N-82 | . | E | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 2.3 | 3.8 | 0.08 |
| N-43 | . | E | . | . | . | . | . | . | . | . | . | . | K | . | . | . | . | . | . | 6.7 | 17.4 | 0.97 |
| N-19 (3) | . | . | . | . | . | . | . | . | . | . | . | M | . | . | . | . | . | . | . | 28.2 | 22.6 | 1.00 |
| N-85 (2) | . | . | . | . | . | . | . | . | . | . | . | D | . | . | . | . | . | . | . | 2.3 | 4.4 | 0.23 |
| N-95 | . | . | . | . | . | . | . | . | . | . | . | D | M | . | . | . | . | . | . | 4.1 | 14.2 | 0.35 |
| N-55 | . | . | . | . | . | . | . | . | . | . | . | D | . | . | . | . | . | . | F | 4.0 | 17.7 | 1.00 |
| N-87 | . | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | L | 10.5 | 15.4 | 0.46 |
| N-70 | . | . | . | . | . | . | . | . | . | . | . | C | K | . | . | . | . | . | . | 20.6 | 16.6 | 1.00 |
| N-120 | . | . | . | . | . | . | . | . | . | . | . | C | D | . | . | . | . | . | L | 5.4 | 15.1 | 0.71 |
| N-31 | . | . | . | Q | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 6.5 | 8.5 | 1.00 |
| N-63 | . | . | . | G | Q | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 4.7 | 11.1 | 1.00 |
| N-74 | . | . | . | . | Q | V | . | . | . | . | . | . | . | . | . | . | . | . | . | 3.5 | 8.6 | 1.00 |
| N-76 | . | S | . | A | Q | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 4.0 | 9.0 | 1.00 |
| N-105 | . | . | . | A | Q | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 3.6 | 3.7 | 0.43 |
| N-117 | . | . | . | . | W | Q | V | . | . | . | . | . | . | . | . | . | . | . | . | 4.7 | 10.3 | 0.52 |
| N-33 (2) | . | . | . | . | . | . | . | . | . | . | . | N | . | . | . | . | . | . | . | 12.7 | 14.9 | 1.00 |
| N-53 | . | . | . | T | . | . | . | . | . | . | . | N | . | . | . | . | . | . | . | 3.4 | 8.7 | 0.96 |

Suppressor mutants

Most of the coprt" mutants found in this study (21 of 29) contain multiple amino acid substitutions. Six of these 21 carry substitutions that by themselves result in a coprt' phenotype: N-70, N-93, N-96, N-98, N-110, and N-119. The other multiple mutants carry substitutions that by themselves score as coprt' or did not occur as single mutants. Although it is tempting to ascribe the coprt' phenotype to specific substitutions in these multiple mutants it may be that only the particular combination of changes is actually responsible for the phenotype.

To assess the likelihood that mutations that result in constitutive coprotease activity will occur at any one of the residues from 152 to 159 we plotted the frequency of coprt' mutants observed as a function of the total number of mutants containing any substitution at each position (Fig. 3). This shows, for example, that 20 mutants contain a substitution at position 157, with 10 being coprt'; 44 contain a substitution at position 158, with 13 being coprt'; and 35 contain a substitution at position 152, with only 4 scoring as coprt'. These data suggest that coprt' mutants are more likely to result from substitution at positions 157 and 158 than other residues in this area. Because Fig. 3 includes multiple as well as single mutants it may actually underestimate the importance of positions 157 and 158 regarding the occurrence of coprt' mutations. Although analysis of single mutants would avoid this problem, we have only a limited number of single substitution mutants at certain positions. Despite this, we note that both single mutants at residue 157 and three of four single mutants at position 158 are coprt'. The difference between the mutant side chains, Cys versus Asp at residue 157 and Lys, Gln and Asn at residue 158, suggests that there is little specificity required for a coprt' mutant at these positions, an observation that is in sharp contrast to positions 155 and 156 (see above). Studies of a larger pool of single mutants at all residues in this region will help clarify this issue.

Second Site Suppressor Mutations—An advantage of using a combinatorial mutagenesis procedure is in the ability to pick up second site suppressor mutations. In this study we have found eight primary single mutants with alterations in DNA repair, coprotease, or both activities that are suppressed by one or more second site mutations (Table V). A number of these second site mutants show differential suppression of the repair versus coprotease activities.

We found two examples of suppressor mutations that corrected severe defects in both the DNA repair and coprotease activities: 1) a secondary Ile155 → Lys change (N-43) corrects the defects in a Lyd5' → Glu mutant (N-82), and 2) a Ser16' → Phe change (N-55) corrects the defects caused by a G~u'~~ → Asp mutation (N-85). A Ser16' → Phe mutation by itself has no detectable effect on either activity (Table I, N-38). For the G~u'~~ → Asp mutation (N-85) we found another second site suppressor, Ile155 → Met (N-95), which corrects only the defective coprotease activity. The Ile155 → Met change by itself results in a very strong constitutive coprotease activity with no effect on DNA repair (Table IV, N-19).

We also obtained several second site mutants that suppressed the constitutive coprotease activity of the original
The final examples of suppressor mutants in Table V again show different effects on the DNA repair versus coprotease activities. A Gly\textsuperscript{157} → Cys substitution (N-87) results in a moderate coprt\textsuperscript{*} mutant and a 50% decrease in DNA repair activity. A secondary Glu\textsuperscript{158} → Lys change (N-70) corrects the DNA repair defect but also increases the level of constitutive coprotease activity. Another mutant that carries two secondary changes suppresses the constitutive coprotease activity and increases, but does not fully restore, the DNA repair activity (N-120).

**DISCUSSION**

By extensively mutagenizing an area of the RecA protein which overlaps the disordered L1 region we have identified specific residues that play important roles in either the recombinational DNA repair or RecA coprotease activities. Considering the recombinational DNA repair activity, the identification of Glu\textsuperscript{154} as the most important residue in this area can be correlated with the position of the side chains for residues 152–156 in the RecA structure. Of these five residues, only Glu\textsuperscript{154} extends inward toward the helical axis of the RecA protein filament (Fig. 4). The remainder of the side chains extend either toward a neighboring monomer or away from the filament structure toward surrounding solvent. However, nearest neighbor analysis of all atoms in the Glu\textsuperscript{154} side chain (maximum interaction distance = 4 Å) reveals no compelling structural reason for the rather strict constraints that we find at this position. It is interesting to speculate, therefore, that the importance of Glu\textsuperscript{154} lies in its interaction with DNA, a constraint that is missing from the current RecA crystal structure (16).

Although there is good evidence that the primary DNA substrate binds deep within the helical groove of the RecA filament away from L1 (31), the positioning of the second DNA when bound to RecA is not yet known. Stoly et al. (16) have speculated that disordered region L2 (residues 196–209) makes up part of the primary DNA site, and L1 residues 157–164 make up part of the secondary DNA site. In Fig. 4 the inner surface of the RecA oligomer is shown for three contiguous subunits. This image shows that although residues within region L2 can create a continuous surface that runs along the inside of the RecA filament, those within region L1 appear to create a continuous surface that runs along the upper part of the protein filament. If the side chain of Glu\textsuperscript{154} plays a role in binding the second DNA substrate, positioning of this DNA should be such that it is readily accessible to the primary DNA, and Glu\textsuperscript{154} might, therefore, be expected to extend toward the helical axis of the protein filament. Because the binding of both DNA substrates to RecA likely occurs via the polyphosphate DNA backbone (32), Glu\textsuperscript{154} may either attenuate DNA binding through repulsive interactions with the DNA backbone or may potentiate binding via cation bridging. Another consideration regarding a DNA binding function for region L1 is that, other than Glu\textsuperscript{154}, it may be the polypeptide backbone atoms that make the contacts with DNA, and the role of the other side chains has to do with positioning these contacts thereby making the identity of the amino acid less important.

Most of the mutants with a substitution at position 154 (18 of 21) are completely inactivated for both repair and coprotease functions. However, the fact that the double mutant Glu\textsuperscript{154} → Asp/Ile\textsuperscript{157} → Met (Table II, N-95) maintains wild type-like coprotease activity but shows a significant inhibition of DNA repair implies that the identity of the side chain at position 154 is not as important for coprotease as for recombinational DNA.
repair activity. Study of a larger population of single mutants at Glu154 will help to resolve this issue.

We performed a nearest neighbor analysis of all atoms in residues 152–156 and found that certain interactions that are seen in the RecA crystal structure are unlikely to be important determinants of function. For example the e-NH$_2$ group of Lys155 is within bonding distance of the Glu156 side chain. However, both of these residues support a variety of substitutions, indicating that this is not a functionally important interaction. The Glu156 side chain is also within H-bonding distance of Tyr158 on the neighboring subunit, but both positions support mutations that eliminate this interaction yet have no inhibitory effect on RecA function (15 and this study). Ile155 is within van der Waals distance of Phe157 on the neighboring subunit. Although the recombination activity of RecA shows a strict requirement for either Phe or Tyr at position 217 (15), Ile155 supports a variety of substitutions, suggesting that this interaction is not essential to RecA function. The crystallographic B-factors are somewhat high for side chain atoms in residues 152 to 156 (range ~36–68), indicating a fair degree of uncertainty regarding their position in the structure. Regardless of the precise positioning, our mutagenesis data indicate that the identity of residue 154 is clearly more important to the recombinational DNA repair activity of RecA than the identity of the other 7 targeted residues.

Analysis of the LexA coprotease activity showed that 20% of all mutants (29 of 149) display a constitutive phenotype. At certain positions there is a strict specificity regarding the substitutions that give rise to coprt$^+$ mutants. For example, the noninduced and induced coprotease activities of the Glu154$ightarrow$Lys mutant are 22.4 and 25.7 (Table 1) in contrast to 5.0 and 10.8 for the Glu156$ightarrow$Arg mutant, demonstrating a dramatic specificity regarding the side chain requirement at this position for constitutive coprotease activity. A similar situation occurs at Ile155, where substitution to Met is the only one of eight recA$^+$ single mutants which effects coprotease function, resulting in strong constitutive activity. In contrast to the specificity observed at positions 155 and 156, a number of different substitutions at Gly157 and Glu158 give rise to coprt$^-$ mutants.

Yu and Egelman (33) recently performed electron microscopic studies of RecA-LexA-DNA complexes and suggest that region L1 is one of the main areas of contact between the RecA and LexA proteins. This raises the possibility that many of the mutations we have found effect coprotease activity by altering part of the LexA binding site. Met and Lys substitutions at residues 155 and 156, respectively, may create new higher affinity interactions with LexA repressor. However, because of their position at the subunit interface in the RecA filament, a specific conformational shift in the oligomeric structure may also contribute to the activity of these mutants. For Gly157 and Glu158 an increase in the affinity for LexA by the removal of bad contacts may underlie the more frequent occurrence of coprt$^-$ mutants at these positions. This could be achieved by removal of the Glu side chain at position 156 or through a more general conformational mechanism resulting from any number of different substitutions at Gly157.

If the L1 region forms part of both the LexA and secondary DNA binding sites one might expect to find an equal number of mutations that disrupt one activity or the other. However, although we found a number of mutants that show an exclusive inhibitory effect on DNA repair, we found no mutants with such an effect on the coprotease activity. These results are consistent with the idea that determinants in the L1 region for DNA binding and catalysis of recombination are significantly more stringent than for LexA binding and repressor autodigestion.

Biochemical studies have been performed on purified mutant RecA proteins that contain either of two different mutations responsible for constitutive coprotease activity; Glu$^+$$ightarrow$Lys

![Fig. 4. Space filling model of three RecA subunits highlighting the disordered regions L1 and L2. This is a cut-away view showing one-half turn of the RecA protein helical oligomer. Main chain carbons are shown in white or yellow for alternating monomers with those of residues 152, 153, 154, 155, and 156 in green. The side chain of Glu154 extends inward toward the helical axis of the RecA filament and is shown in green in all three subunits. Side chains of other residues are not shown. Residues that flank disordered region L1 (Glu156 and Gly157) are shown in green and labeled with a $^+$, residues that flank disordered region L2 (Ile155 and Thr156) are shown in red and labeled with a 2. ADP is in violet.](image-url)
Mutagenesis of RecA Region L1

FIG. 5. Position of copr\textsuperscript{c} mutations in the RecA protein structure. This image shows one turn (six subunits) of two neighboring RecA oligomers as seen in the crystal structure (16). The central boxed area corresponds to the interfilament contact region defined by Story et al. (16). This region contains several residues at which mutations lead to a copr\textsuperscript{c} phenotype (Glu\textsuperscript{265} → Lys, Thr\textsuperscript{269} → Ile, Glu\textsuperscript{344} → Lys, or Gly\textsuperscript{391} → Asp or Ser) or impose a temperature dependence on copr\textsuperscript{c} activity (Ile\textsuperscript{79} → Val). Specific mutations at Ile\textsuperscript{79} and Glu\textsuperscript{344} (see "Discussion"), as well as a Arg\textsuperscript{344} → Cys mutation also result in copr\textsuperscript{c} mutants. These 3 residues are highlighted in all six subunits in each filament and are clearly distant from the interfilament contact region (labels appear in only the topmost subunits in panel A). Panel A, side view. Panel B, top view down the 6-fold central axis.
We have isolated a number of second site suppressor mutations that effect either or both the DNA repair and coprotease activities. The occurrence of a relatively large number of suppressors in a small, defined area suggests a functional relationship between residues in this region of the protein structure, supporting the idea that this area forms part of the RecA and secondary DNA binding sites. Also, the fact that some of these suppressors differentially effect the recombination repair versus coprotease activities reinforces the idea that these functions, although overlapping, are clearly separable.

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