The Conserved Lys-95 Charged Residue Cluster Is Critical for the Homodimerization and Enzyme Activity of Human Ribonucleotide Reductase Small Subunit M2*

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Ribonucleotide reductase (RR) catalyzes the reduction of ribonucleotides to deoxyribonucleotides for DNA synthesis. Human RR small subunit M2 exists in a homodimer form. However, the importance of the dimer form to the enzyme and the related mechanism remain unclear. In this study, we tried to identify the interfacial residues that may mediate the assembly of M2 homodimer by computational alanine scanning based on the x-ray crystal structure. Co-immunoprecipitation, size exclusion chromatography, and RR activity assays showed that the K95E mutation in M2 resulted in dimer disassembly and enzyme activity inhibition. In comparison, the charge-exchanging double mutation of K95E and E98K recovered the dimerization and activity. Structural comparisons suggested that a conserved cluster of charged residues, including Lys-95, Glu-98, Glu-105, and Glu-174, at the interface may function as an ionic lock for M2 homodimer. Although the measurements of the radical and iron contents showed that the monomer (the K95E mutant) was capable of generating the diiron and tyrosyl radical cofactor, co-immunoprecipitation and competitive enzyme inhibition assays indicated that the disassembly of M2 dimer reduced its interaction with the large subunit M1. In addition, the immunofluorescent and fusion protein-fluorescent imaging analyses showed that the dissociation of M2 dimer altered its subcellular localization. Finally, the transfection of the wild-type M2 but not the K95E mutant rescued the G1/S phase cell cycle arrest and cell growth inhibition caused by the siRNA knockdown of M2. Thus, the conserved Lys-95 charged residue cluster is critical for human RR M2 homodimerization, which is indispensable to constitute an active holoenzyme.

Ribonucleotide reductase (RR) is a multisubunit enzyme responsible for the reduction of ribonucleotides to corresponding deoxyribonucleotides, which are building blocks for DNA replication and repair in all living cells. Therefore, the enzyme is critical for the control of cell proliferation and the maintenance of genome stability. The identified regulatory mechanisms for RR activity in cells mainly include gene transcription, protein degradation, allosteric regulation, and subcellular translocation (1–3). A failure in the control of the levels and/or balances of deoxyribonucleotides leads to genetic abnormalities, cancers, or cell death (4, 5). Three main classes of RR have been described, based on the metal cofactors for the catalytic activity. The class I enzymes require dinuclear metal clusters for activity: an FeIII/FeII-tirosyl radical (Y') cofactor (class Ia), a MnII/MnII-Y' cofactor (class Ib), and a MnIV/FeII cofactor (class Ic) (6, 7). Class Ia RRs are found in all types of eukaryotes (including human, mouse, and yeast), a few prokaryotes (including Escherichia coli), several viruses, and some bacteriophages. This subclass of enzymes is composed of an R1(αα)R2(ββ) monosubunit protein complex. The large subunit R1 harbors the active site and allosteric sites (1, 8), and the small subunit R2 houses a diiron-tirosyl radical cofactor essential for generating the active site cysteinyl radical in R1 via a proton-coupled electron transport chain between the two subunits (3, 9). The C-terminal tail of R2 interacts with R1 to form a holoenzyme (10, 11).

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The abbreviations used are: RR, ribonucleotide reductase; SASA, solvent-accessible surface area; MM/PBSA, molecular mechanics/Poisson-Boltzmann surface area; MD, molecular dynamics; PDB, Protein Data Bank; TRITC, tetramethylrhodamine isothiocyanate; EYFP, enhanced yellow fluorescent protein.
Lys-95 Cluster Critical for M2 Dimerization and Activity

There exist different oligomeric states of class Ia RRs, and a common active form has been proposed to be a transient \((\alpha_2\beta_2)_6\) complex (3, 12), which is allosterically regulated by the nucleoside triphosphates (13). Under physiological conditions, E. coli RR exists as a mixture of transient \(\alpha_2\beta_2\) and \(\alpha_6\beta_4\) species whose distributions are modulated by allosteric effectors (12, 14). The yeast Saccharomyces cerevisiae RR comprises \(\alpha\) and \(\beta'\) subunits in an \((\alpha_2\beta_2)_6\) active holoenzyme (15–17).

Mammalian genomes contain a single R1 gene (also named as M1 in human RR) and two R2 genes for R2 and its homologue p53R2 (also named as M2 and M2B, respectively, in human RR). For eukaryotic RRs, the ATP/dATP-induced R1 multimers can interact with R2 dimers to form active \(\alpha_2\beta_2\), \(\alpha_6\beta_2\), or \(\alpha_6\beta_6\) complexes or inhibited \(\alpha_6\beta_2\) complexes (8, 18–20).

It seems that the dimer form of R1 and R2 is a basic entity in an active RR holoenzyme. R1, monomeric in the absence of ligands, dimerizes in the presence of substrate or effectors (21). When the allosteric substrate specificity effectors bind to one R1 monomer, a minor conformational change is induced in a connecting loop that influences binding of the correct substrate to the second R1 monomer. Therefore, R1 dimer formation is a prerequisite to function properly (18, 22). R2 forms a homodimer in cells (23, 24). However, using heterodimers containing deuterated tyrosine on the full-length side and protonated tyrosine on the truncated side, Sjöberg et al. (25) found that the tyrosyl radical is randomly generated in either of the two polypeptide chains of the heterodimeric R2 subunit in E. coli. In a recent study on S. cerevisiae RR, Zhang et al. (17) demonstrated that the C-terminal tails of \(\beta'\) interact only with the proximal \(\alpha\) within each \((\alpha/\beta')\) pair in the holoenzyme. Although each monomer of R2 from E. coli, mouse, or human possesses a diiron-tyrosyl radical site and a set of residues for the radical transfer to M1, one monomer probably can function independently for the radical generation and R1 binding to form an active complex. In this case, however, the contribution of the dimer form of the small subunit to the function of a class Ia RR holoenzyme is a fundamental biological question.

In this study, we identified a conserved cluster of charged residues, including Lys-95, Glu-98, Glu-105, and Glu-174, at the homodimer interface in human RR small subunit M2 based on its x-ray crystal structure in combination with site mutagenesis and functional analysis. We demonstrated that the conserved Lys-95 charged residue cluster is critical for M2 homodimer assembly, which is indispensable to constitute an active holoenzyme and function in cells.

**EXPERIMENTAL PROCEDURES**

**Prediction of Dimer Interface Residues Based on M2 Structure**—The M2 crystal structure was retrieved from the protein data bank (PDB code 3OLJ) (Fig. 1A). First, solvent-accessible surface areas (SASAs) for each residue of the dimer were calculated. Second, the dimer was split into two pieces, one for each protomer. Third, the protomer-only SASA for each residue was calculated. Last, the difference between the dimer-based SASA and the protomer-only-based SASA was calculated for each residue. If the value was greater than 0.75 Å², it was defined as an interface residue (Fig. 1B). The calculations were performed with a PyMOL script.

**Computational Alanine Scanning**—Alanine, glycine, and proline are not suitable for computational alanine scanning. Except for these residues, each interface residue predicted above was mutated to alanine (26). The binding free energies \((\Delta G_{\text{bind}})\) of the wild-type and alanine mutants were calculated using the molecular mechanics/generalized Born surface area (MM/GBSA) (27) approach and molecular mechanics/Poisson-Boltzmann surface area (MM/PBSA) (28) approach, which are programmed in the AMBER9 package. The energy difference between the mutant and the wild-type M2 \((\Delta G_{\text{mut}} - \Delta G_{\text{WT}})\) was calculated as \(\Delta G_{\text{mut}} - \Delta G_{\text{WT}}\). \(\Delta G_{\text{mut}} - \Delta G_{\text{WT}}\) is a measure of the contribution of the interface residues to M2 dimerization. A larger absolute value indicates a higher contribution.

To carry out computational alanine scanning, a molecular dynamics (MD) simulation was performed to obtain a set of equilibrium conformations of the dimer. MD simulations were carried out for M2 dimer structure using the AMBER9 package (29) with the ff03 AMBER force field (30). After removing alternative conformations, the protonation states of histidine were assigned manually. The M2 dimer was solvated by a rectangular box of TIP3P (31) waters extended at least 10.0 Å in each direction from the dimer. Then sodium ions were added to generate a neutral simulation system. 2000 steps of minimization were performed in order to minimize the system with the protein restrained, Cα restrained, and the whole system relaxed. The system was heated up in two steps using a Langevin temperature equilibration scheme with restraints on the protein. First, with the backbone restrained, a constant volume periodic boundary was used to heat the system from 0 to 300 K in 20 ps. Second, with Cα restrained, a constant pressure periodic boundary was used to equilibrate the system at 300 K over a time scale of 100 ps. Finally, a 20-ns MD equilibration was run on the whole system with constant pressure at 300 K. The Langevin dynamics was used to control the temperature at 300 K with a collision frequency of 2.0 ps⁻¹. A pressure-coupling algorithm was used to maintain the pressure with a relaxation time of 2 ps. The SHAKE algorithm (32) was used to constrain bonds involving hydrogen, which allowed for a 2-fs time step during the heating and equilibration phases, and the particle-mesh Ewald algorithm (33, 34) was used to calculate long distance electrostatic interactions with a cut-off of 10.0 Å.

It was found that the system was equilibrated after 10 ns (Fig. 1C). A total of 200 snapshots were saved during the equilibration stage for computational alanine-scanning analysis, one snapshot per 50 ps of dynamic simulation. The binding free energy differences \((\Delta G_{\text{mut}} - \Delta G_{\text{WT}})\) between the mutants and the wild-type M2 \((\Delta G_{\text{mut}} - \Delta G_{\text{WT}})\) from MM/GBSA calculations are shown in Fig. 1D, whereas the detailed data are presented in Table 1. A positive \(\Delta G_{\text{mut}} - \Delta G_{\text{WT}}\) value implies a positive contribution to the M2 dimerization.

**Plasmid Construction**—pET28a-M2 and pET28b-M1 constructs for the full-length small and large subunit proteins of human RR were prepared previously (35). The coding sequence of M2 was inserted into pcDNA3.1 vector with a FLAG tag at the N terminus, a 2× HA tag at the C terminus, or a 6× Myc tag at the C terminus and also inserted into pEYFP vector with an EYFP tag at the C terminus. The coding sequence of M1 was
Table 1

| M2     | ΔG_{bind, GB} | ΔG_{mut} - WT, GB | ΔG_{bind, PB} | ΔG_{mut} - WT, PB |
|--------|---------------|--------------------|---------------|-------------------|
| R79A   | 28            | 27                 | 14            | 37                |
| E147A  | 35            | 20                 | 28            | 23                |
| R159A  | 43            | 13                 | 4             | 47                |
| K95A   | 43            | 13                 | 37            | 15                |
| F83A   | 44            | 11                 | 48            | 8                 |
| F80A   | 45            | 1                   | 47            | 5                 |
| R78A   | 48            | 7                  | 41            | 11                |
| F101A  | 49            | 6                  | 49            | 3                 |
| Y94A   | 50            | 6                  | 57            | 5                 |
| I82A   | 50            | 5                  | 52            | 0                 |
| F194A  | 50            | 5                  | 48            | 4                 |
| W91A   | 51            | 4                  | 53            | 2                 |
| F164A  | 52            | 4                  | 51            | 1                 |
| V81A   | 52            | 3                  | 48            | 4                 |
| N170A  | 53            | 3                  | 50            | 2                 |
| Q151A  | 53            | 2                  | 49            | 2                 |
| H166A  | 53            | 2                  | 48            | 3                 |
| I171A  | 53            | 2                  | 54            | 3                 |
| T103A  | 54            | 2                  | 48            | 4                 |
| I197A  | 54            | 1                  | 50            | 1                 |
| S150A  | 54            | 1                  | 52            | 0                 |
| N195A  | 55            | 1                  | 51            | 1                 |
| C160A  | 55            | 0                  | 53            | 1                 |
| L178A  | 55            | 0                  | 52            | 0                 |
| WT     | 55            | 0                  | 52            | 0                 |
| E174A  | 56            | 0                  | 58            | 6                 |
| N142A  | 56            | 1                  | 52            | 1                 |
| S173A  | 56            | 1                  | 52            | 0                 |
| E143A  | 56            | 1                  | 53            | 2                 |
| S177A  | 56            | 1                  | 53            | 2                 |
| E198A  | 57            | 1                  | 52            | 0                 |
| V146A  | 57            | 2                  | 51            | 1                 |
| E191A  | 58            | 3                  | 54            | 2                 |
| D181A  | 58            | 3                  | 55            | 4                 |
| E105A  | 60            | 5                  | 59            | 7                 |
| E98A   | 63            | 7                  | 67            | 16                |

*GB, MM/GBSA.
*PB, PB/GBSA.

Table 2

| Plasmid         | Primer used for construction of M2 and M1 protein expression plasmids | Sequence                      |
|-----------------|-----------------------------------------------------------------------|-------------------------------|
| pcDNA3.1-HA-M2(WT) | F(Xhol)                                                               | 5'-ACGCTTCGAGTCATGTCCTCCCTTCCTAGTCGTCGTC-3' |
| pcDNA3.1-c-Myc-M2(WT)/FLAG-M2(WT) | F(BamHI)                                                             | 5'-ATCCGGATCCCAAGATTCAGATCATCCAAAGGTA-3' |
| pcDNA3.1-c-Myc-M2(WT) | F(Xhol)                                                               | 5'-ACGCTTCGAGTCATGTCCTCCCTTCCTAGTCGTCGTC-3' |
| pcDNA3.1-c-Myc-M2(WT) | F(BamHI)                                                             | 5'-ATCCGGATCCCAAGATTCAGATCATCCAAAGGTA-3' |
| pEYFP-M2(WT)      | R(Xhol)                                                               | 5'-ATCCGGATCCCAAGATTCAGATCATCCAAAGGTA-3' |

Table 3

| Mutant          | Primer sequence (forward)                                          |
|-----------------|---------------------------------------------------------------------|
| R78A            | AGAGAAAAACCGCGGCTTGGTACATCCTCCCTCCCTCCCTAGTCGTCGTC-3' |
| R78E            | GCTGCTTCGAGAAAACCGCGGCTTGGTACATCCTCCCTCCCTCCCTAGTCGTCGTC-3' |
| R79A            | GAGAGAAAAACCGCGGCTTGGTACATCCTCCCTCCCTCCCTAGTCGTCGTC-3' |
| F80A            | AGAGAAAAACCGCGGCTTGGTACATCCTCCCTCCCTCCCTAGTCGTCGTC-3' |
| F83A            | CGCTTGGTACATCCTCCCTCCCTCCCTCCCTAGTCGTCGTC-3' |
| K95A            | CGCTTGGTACATCCTCCCTCCCTCCCTCCCTAGTCGTCGTC-3' |
| K95Q            | CGCTTGGTACATCCTCCCTCCCTCCCTCCCTAGTCGTCGTC-3' |
| E98A            | CGCTTGGTACATCCTCCCTCCCTCCCTCCCTAGTCGTCGTC-3' |
| E98K            | CGCTTGGTACATCCTCCCTCCCTCCCTCCCTAGTCGTCGTC-3' |
| E98K/K95E       | CGCTTGGTACATCCTCCCTCCCTCCCTCCCTAGTCGTCGTC-3' |
| F101A           | CGCTTGGTACATCCTCCCTCCCTCCCTCCCTAGTCGTCGTC-3' |
| E105A           | CGCTTGGTACATCCTCCCTCCCTCCCTCCCTAGTCGTCGTC-3' |
| E147A           | CGCTTGGTACATCCTCCCTCCCTCCCTCCCTAGTCGTCGTC-3' |
| E147R           | CGCTTGGTACATCCTCCCTCCCTCCCTCCCTAGTCGTCGTC-3' |
| E191A           | CGCTTGGTACATCCTCCCTCCCTCCCTCCCTAGTCGTCGTC-3' |
| R264Q           | CGCTTGGTACATCCTCCCTCCCTCCCTCCCTAGTCGTCGTC-3' |

Boldface letters denote the mutated codons.
Lys-95 Cluster Critical for M2 Dimerization and Activity

molecular weight and oligomeric state of the proteins were estimated based on the standard curve generated by a Bio-Rad molecular mass marker kit, which included thyroglobin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa).

Co-immunoprecipitation and Immunoblotting Analysis—For interaction between HA-M2 and c-Myc-M2, the cell lysates were prepared at 48 h after transfection using radioimmunoprecipitation assay buffer containing 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 g/ml leupeptin, 10 g/ml aprotinin, 1 mM vanadate, and 1 mM NaF. The lysates were clarified by centrifugation at 13,000 rpm for 20 min. Then the supernatant was incubated overnight at 4 °C with 4 µl of anti-mouse c-Myc (Santa Cruz Biotechnology, Inc.). The mixture was then incubated with 20 µl of 50% protein G Plus-agarose (Santa Cruz Biotechnology, Inc.) for 4 h at 4 °C with gentle rocking. After three washes in radioimmunoprecipitation assay buffer, the immunoprecipitates were separated on SDS-PAGE (10%), transferred to nitrocellulose membrane, and blocked with TBS containing 5% (w/v) skim milk and 0.1% Tween 20. Primary antibodies included rabbit anti-human HA, or rabbit anti-human c-Myc (both from Santa Cruz Biotechnology, Inc.). Membranes were incubated with the primary antibody overnight at 4 °C, washed three times with TBS containing 0.1% Tween 20, and then stained with Alex 680- or IR 800-conjugated secondary antibody (LI-COR, Lincoln, NE) for LI-COR analysis.

For interaction between FLAG-M2 and c-Myc-M1, the cell lysis buffer was as follows: 1× phosphate-buffered saline (PBS), 1% Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM PMSF, 10 g/ml leupeptin, 10 g/ml aprotinin, 1 mM vanadate, and 1 mM NaF (37). Proteins were immunoprecipitated with rabbit anti-human c-Myc antibody and detected by immunoblotting using rabbit anti-human FLAG antibody (Santa Cruz Biotechnology, Inc.).

RR Activity Assay and Competitive Inhibition—The CDP reduction activity of the recombinant RR proteins was assayed according to our previous procedure (35, 38). The reaction mixture contained 0.125 μM [3H]CDP, 50 mM HEPES (pH 7.2), 100 mM KCl, 6 mM DTT, 4 mM magnesium acetate, 2 mM ATP, 0.05 mM CDP, and mixed recombinant proteins of M2 and M1 at a 1:1 ratio (each 0.5–1 μM) in a final volume of 100 µl. After incubation at 37 °C for 15–30 min and dephosphorylation, samples were analyzed by HPLC and liquid scintillation counting. For competitive inhibition of the enzyme activity, the M2 mutant protein (R264Q or K95E) was serially diluted in 25 mM Tris-HCl (pH 7.4) and incubated with the wild-type M2 and M1 proteins at room temperature for 30 min. Then the RR activity was analyzed as described above.

Cellular RR activity assays were performed as described previously (39). Briefly, cultured cells were lysed in a low salt homogenization buffer (10 mM HEPES, pH 7.2, and 2 mM DTT) by 20 passages through a 27-gauge needle on ice. Then the same volume of high salt buffer (1 mM HEPES, pH 7.2, 2 mM DTT) with protease inhibitors was added and passed through the needle again. The supernatants were collected after centrifugation, and proteins were eluted by gel size exclusion (Sephadex G-25, GE Healthcare). The protein concentration and the RR activity in the cellular extracts were determined as above. RR activity = dCDP/C(P + dCDP) × 100%.

Electron Paramagnetic Resonance (EPR) Measurements—The wild-type and mutant M2 proteins were regenerated as described previously (38, 40, 41). Briefly, a solution containing 1.5 mM ferrous iron (FeCl2) and 200 mM DTT was added to the fresh purified R2 protein solutions in 50 mM Tris-HCl (pH 7.4) and 0.1 mM KCl to give a final concentration of 10 mM DTT and 75 µM ferrous iron. Then air was bubbled through the solution, and the mixture was incubated for 15 min at 25 °C. The protein was passed through gel filtration on a Sephadex G25 column. After incubation at room temperature for different intervals, the protein samples were frozen in liquid nitrogen, and the X-band EPR spectra were measured with a Bruker EMX spectrometer equipped with an Oxford helium cryostat. The instrumental parameters were as follows: T = 30 K; microwave frequency, 9.376 GHz; microwave power, 0.5 milliwatt; modulation amplitude, 4 Gauss; and modulation frequency, 100 kHz (35, 38). Radical concentrations were determined by a comparison with a standard sample of 1 mM Cu2+, 10 mM EDTA.

Inductively Coupled Plasma Optical Emission Spectrometry—The iron content of the regenerated wild-type and mutant M2 proteins was measured using a PerkinElmer Life Sciences inductively coupled plasma optical emission spectrometer (Optima 8000DV, PerkinElmer Life Sciences) after acid digestion of the samples. Pure iron standard solution (Shanghai Institute of Measurement and Testing Technology) was serially diluted from 10 to 1000 mg/liter to construct the standard curve (y = 183,770x; x, concentration; y, absorbance; R2 = 1).

Indirect Immunofluorescence Microscopy—The cells were fixed in 4% (w/v) paraformaldehyde and permeabilized with 0.2% Triton X-100. Then the samples were blocked with 1% (w/v) bovine serum albumin, stained in sequence with mouse anti-FLAG antibody (Sigma), TRITC-conjugated secondary antibody, and DAPI (42). The cells were visualized using a confocal microscope (Zeiss LSM 510 META).

Imaging of EYFP Fusion Proteins—HeLa cells were transiently transfected with either the wild-type or a mutant M2 construct carrying EYFP marker. Cells were imaged 48 h after transfection using a Nikon Eclipse Ti-S microscope.

Cell Cycle Analysis—HeLa cells were harvested, fixed in cold 70% ethanol, and stained with propidium iodide solution after transfection with the human RR M2 gene-specific siRNA (Santa Cruz Biotechnology, Inc.) and plasmid constructs encoding the wild-type or K95E mutant M2 for 48 h. The scramble siRNA (Santa Cruz Biotechnology, Inc.) and empty vector were used as controls. The cell cycle was analyzed by flow cytometry, and the data were analyzed using the WinMDI program.

Cell Proliferation Assays—HeLa cells were plated in 96-well plates at 2 × 104 cells/well. After transfection for 72 h, cell viabilities were assayed using a cell-counting kit (CCK-8, Dojindo (Kumamoto, Japan)).
RESULTS

Identification of the Amino Acid Residues That Mediate M2 Dimerization—Human RR small subunit M2 is a homodimer, and the dimer interface can be divided into two identical interaction sites (i.e., A1 interacts with B2 and A2 interacts with B1, where A1 is identical to B1 and A2 is identical to B2) based on the M2 crystal structure (PDB code 3OLJ) (Fig. 1A). Based on the SASA difference between the dimer and protomer, 42 residues were identified at the interface of the M2 dimer: Pro-77, Arg-78, Arg-79, Phe-80, Val-81, Ile-82, Phe-83, Pro-84, Trp-91, Tyr-94, Lys-95, Glu-98, Phe-101, Thr-103, Ala-135, Ala-136, Gly-139, Asn-142, Glu-143, Val-146, Glu-147, Ser-150, Gln-151, Arg-159, Cys-160, Gly-163, Phe-164, Ile-166, Ala-167, Asn-170, Ile-171, Ser-173, Glu-174, Ser-177, Leu-178, Asp-181, Glu-191, Phe-194, Asn-195, Ile-197, and Glu-198 (Fig. 1B). After removing alanine, glycine, and proline, which are not suitable for computational alanine scanning, 35 residues were left and used to estimate the binding free energy changes as a result of mutating the interfacial residues to alanine. To carry out computational alanine scanning, a MD simulation was performed to obtain a set of equilibrium conformations of the dimer. It was found that the system was equilibrated after 10 ns (Fig. 1C). The computational alanine scanning by MM/GBSA suggested that Arg-79, Glu-147, Arg-159, Lys-95, Phe-83, and Arg-78 contribute significantly to M2 dimerization (Fig. 1D and Table 1). Similar results were also obtained from MM/PBSA calculations (Table 1). Combined analysis with the crystal structure of M2 dimer suggested that three clusters of interfacial charged residues might be responsible for the dimerization (Fig. 1E) (i.e., Arg-78/Glu-191 or Arg-78/Glu-191, Arg-79/Glu-147/Arg-159 or Arg-79/Glu-147, Arg-79/Glu-147/Arg-159, and Lys-95/Glu-98/Glu-105/Glu-174 or Lys-95/Glu-98/Glu-105/Glu-174 (the residues in protomer 1 or protomer 2 are distinguished by superscript number 1 or 2, respectively)). In addition, several hydrophobic residues at the interface of the M2 dimer, such as Phe-80, Phe-83, and Phe-101, might also contribute to dimer formation.

The K95E Mutation Prevents M2 Dimerization and Inhibits RR Activity—To test whether the above identified residues are responsible for M2 dimerization, a series of expression plasmids for the wild-type and mutant M2 proteins were constructed. The charge-opposite mutants for the three clusters of interfacial charged residues included R78E, K95E, and E147R. The alanine mutants included R78A, R79A, F80A, K95A, E147A, R159A, E174A, E191A, and E98A. After co-transfecting HA-M2 and c-Myc-M2 into HEK 293T cells, co-immunoprecipitation experiments were performed to determine the formation of M2 homodimers. The results showed that the K95E mutation prevented M2 dimerization and inhibited RR activity (Fig. 2A).

Lys-95 Cluster Critical for M2 Dimerization and Activity—To further confirm the importance of the Lys-95 cluster in M2 dimerization, a series of expression plasmids for the wild-type and mutant M2 proteins were constructed. The charge-opposite mutants included R78E, K95E, and E147R. The alanine mutants included R78A, R79A, F80A, K95A, E147A, R159A, E174A, E191A, and E98A. After co-transfecting HA-M2 and c-Myc-M2 into HEK 293T cells, co-immunoprecipitation experiments were performed to determine the formation of M2 homodimers. The results showed that the K95E mutation prevented M2 dimerization and inhibited RR activity (Fig. 2B). The combined results suggested that the Lys-95 cluster is critical for M2 dimerization and activity.
cipitation experiments showed that the interaction between either the two K95E mutants or the two K95A mutants was reduced significantly compared with that between the wild-type or other mutant M2 proteins (Fig. 2A). The RR activity assays with the recombinant M2 and M1 proteins (Fig. 2B) showed that the K95E mutation in M2 sharply attenuated the enzyme activity to 4.9 ± 2.1% compared with the wild-type protein (Fig. 2C). The size exclusion chromatography experiments using the recombinant M2 proteins showed that the wild-type M2 ran as a dimer, whereas the K95E mutant was mainly in a monomer form (Fig. 2D). Furthermore, the overexpression of wild-type M2 in KB cells markedly increased the intracellular RR activity, but the K95E mutant failed (Fig. 2E).

The possible role of hydrophobic interactions in the dimer assembly was also examined. The RR activity and M2 dimer formation were affected by the F83A and F101A mutations but much less than by the K95E/A mutation (Fig. 2C and D). The F80A mutation significantly inhibited the enzyme activity (Fig. 2C); however, both the co-immunoprecipitation and size exclusion chromatography experiments indicated little interference in the dimer formation by the mutation (Fig. 2C and D). The above results suggested that Lys-95 plays an important role in M2 dimerization, and the Lys-95/Glu-98/Glu-105/Glu-174 cluster (hereafter referred to as the Lys-95 charged residue cluster) is probably a key interfacial site for the dimer assembly. In addition, the hydrophobic residues at the interface may also aid the dimer formation.

The Charge-exchanging Co-mutation of K95E and E98K Recovers M2 Dimerization as Well as RR Activity—In the Lys-95 charged residue cluster, only Lys-95 is a positively charged residue. The double mutations of K95E and E98K for charge-exchanging between the two residues were introduced into the Lys-95 charged residue cluster. After transfecting into HEK 293T cells, co-immunoprecipitation experiments showed that the K95E/E98K double mutant, which exchanged the charge property between Lys-95 and Glu-98, restored the interaction between the two monomers compared with the K95E single mutation (Fig. 3A). The K95Q mutant, which introduced a glutamine into the Lys-95 charged residue cluster, interfered less with the dimerization than the K95E mutant.

Size exclusion chromatography and RR activity assays were performed using the recombinant RR proteins (Fig. 3B). The gel filtration examinations showed that the K95E/E98K double mutations and the K95Q mutation fully and partially recovered

FIGURE 2. A, HEK 293T cells were co-transfected with the expression plasmids for the wild-type and mutant M2 proteins with a HA or c-Myc tag as indicated. The cell lysates were prepared at 48 h after transfection, and the co-immunoprecipitations (IP) were performed with anti-c-Myc antibody followed by immunoblotting detection with anti-HA antibody. Ten percent of the cell lysate for each co-immunoprecipitation was used for the input control. B, SDS-PAGE (10%) analysis of the purity and homogeneity of the His-tagged recombinant M2 and M1 proteins purified by nickel resin affinity chromatography. C, the recombinant M2 and M1 proteins were mixed and assayed for the CDP reduction activity, as described under Experimental Procedures. The enzyme activity of each mutant M2 protein is presented as a percentage of that for the wild type. D, size exclusion chromatography measurements were conducted for the recombinant wild-type and mutant M2 proteins. The protein elution using 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 2 mM DTT was monitored by absorbance at 280 nm with a set of molecular mass markers. E, KB cells were transfected with empty vector (EV) and the wild-type and K95E mutated M2 plasmids, respectively. The expressions of the M2 proteins in KB cells were shown using immunoblotting examination. The relative RR activity in the cellular lysates was measured as above. Error bars, S.D.
The dimerization of M2, respectively, compared with the K95E mutation and the K95E/E98A double mutations (Fig. 3C). The RR activity assays further confirmed that whereas the K95E mutation almost quenched the enzyme activity, the K95Q mutant and the K95E/E98K double mutant possessed 55.9 ± 5.3 and 99.0 ± 4.9% of the wild-type M2 activity, respectively (Fig. 3D). The results suggested that the Lys-95 charged residue cluster couples the two monomers across the interface most likely through an “ionic lock”-like mechanism, where the positively charged Lys-95 plays a key role in balancing the negative charges from the other three glutamic acid residues (Glu-98, Glu-105, and Glu-174).

The Lys-95 Charged Residue Cluster Is Conserved in the Small Subunits of Class Ia RR—To investigate whether the charge interaction in the Lys-95 cluster site is conserved in class Ia RR small subunits, structural superposition analysis was performed between human M2, human p53R2, mouse R2, E. coli R2, yeast RNR2-RNR4 heterodimer, and yeast RNR2-RNR2 homodimer, respectively (Fig. 4). It was shown that the Lys-95 cluster is highly conserved among the small subunits of human, mouse, and yeast RRs. In the eukaryotic RR small subunits, a conserved water is found in the charged residue cluster, which may contribute to the stabilization of the cluster by mediating a hydrogen bond network. Despite the low sequence identity of 20% between human M2 and E. coli R2, it seems that the charged residue cluster still exists with Lys-95 substituted by Glu-41-E. coli R2 and Glu-174-R2 substituted by Arg-120-E. coli R2, respectively; in addition, Glu-37-E. coli R2, corresponding to Trp-91-M2, probably plays a role as Glu-98-M2 for the charge balance at the interface. Thus, the “ionic lock” mechanism for the assembly of M2 dimer is probably conserved in class Ia RR small subunits.

The K95E Mutation Does Not Interfere with the Generation of the Diiron and Tyrosyl Radical Cofactor in M2—The diiron and tyrosyl radical cofactor in M2 is essential for RR activity. Structural analysis suggested that the Lys-95 mutations should not affect the diiron site or tyrosyl radical site within the same protomer because the distances are 25.3 and > 15.3 Å between Lys-95 and the radical residue Tyr-176 and the iron ligand residues, respectively (Fig. 5D). However, Glu-174, one of the Lys-95 cluster residues, resides on the same α helix as the radical residue Tyr-176 and the iron ligand residue His-172, with only a one-residue interval, raising a possibility that the mutations on Lys-95 may affect the essential residues Tyr-176 and His-172 through interfering with Glu-174 (Fig. 5A). However, EPR analyses showed that the K95E mutant maintained the tyrosyl radical to a similar extent as wild-type M2 at different time points up to 120 min at room temperature (Fig. 5B and Table 4). Inductively coupled plasma optical emission spectrometry measurements showed that the iron content of the wild-type and K95E mutant M2 proteins was similar (Table 4). The results suggested that the M2 monomer (the K95E mutant) was capable of generating and maintaining the diiron and tyrosyl radical cofactor, although it failed to constitute an active RR. This also excluded the possibility that the quenching of the RR activity in the K95E mutant resulted from interfering with the essential cofactor formation and stability.
The K95E Mutation in M2 Affects Its Interaction with M1 and Localization in Cells—Co-immunoprecipitation was employed to examine whether the disassociation of M2 dimer by the K95E mutation affects the interaction between the small and large subunits of RR. The result showed that the binding between the K95E mutant M2 and the wild-type M1 was significantly decreased in the co-transfected cells in comparison with the wild-type M2 (Fig. 6A). Arg-265-mouse R2 is conserved in all known sequences of the small subunits of class Ia RRs. The R265Q mutation in mouse R2 depleted its RR activity but had minor effects on tyrosyl radical generation and the binding of M2 to M1 (43). The R264Q mutant M2 (corresponding to Arg-265-mouse R2) was used for competitive enzyme inhibition assays (Fig. 6B). The result showed that the R264Q-M2 was capable of competing with the wild-type M2 to bind to M1 and hence inhibiting the enzyme activity. In comparison, the K95E mutant exhibited a much lower competitive ability to inhibit the enzyme activity, reflecting that the K95E mutant possessed a significantly reduced ability to bind M1. The results suggested that the disruption of M2 dimer reduced its interaction with M1 to form an active holoenzyme in cells.

Human RR subunits, including M2, are mainly distributed in the cytoplasm under normal growth conditions (44). The nuclear localization of RR subunits is associated with dynamic changes in RR enzymatic activity and nuclear proportions of dNTPs at the G1/S phase transition (37, 44). Indirect immunofluorescence confocal microscopy (Fig. 6C) and EYFP fusion protein-fluorescent imaging (Fig. 6D) showed that whereas the wild-type M2 was predominantly located in the cytoplasm and nucleus, and the K95E/E98K double mutants reversed the disordered distribution. The results suggested that the dimer form of M2 is important for its normal subcellular distribution.

The K95E Mutant Fails to Rescue the G1/S Phase Cell Cycle Arrest and Cell Proliferation Inhibition Induced by M2 siRNA Knockdown—During the cell cycle, M2 is only expressed in the late G1/early S phase for DNA replication. Inhibition of M2 prevents cell cycle progression and cell proliferation. Flow cytometry analysis showed that the transfected expression of the wild-type M2 reversed the G1/S phase cell cycle arrest induced by the siRNA knockdown of M2, whereas the K95E mutant did not (Fig. 7A). Furthermore, cell proliferation assays showed that the transfection of the wild-type M2 but not the K95E mutant rescued the cell growth (Fig. 7B). The corre-
Lys-95 Cluster Critical for M2 Dimerization and Activity

The small subunits of class Ia RRs are dimeric in vitro and in cells (45–47). However, it is still unclear how the two protomers interact with each other and whether each protomer can function as an independent unit for RR activity. In this study, structure-based molecular dynamics simulations and alanine scanning analysis suggested that three clusters of charged residues [Arg-781/Glu-1912, Arg-791/Glu-1472/Arg-1591, and Lys-951/Glu-981/Glu-1052/Glu-1742] at the homodimeric interface may mediate the assembly of human RR small subunit M2 (Fig. 1). Site-directed mutagenesis combined with co-immunoprecipitation of the M2 proteins in the transfected cells and size exclusion chromatography analysis using the purified M2 proteins showed that the Lys-95 charged residue cluster (Lys-951/ Glu-981/Glu-1052/Glu-1742) played a pivotal role in the dimerization (Fig. 2, A and D). RR activity assays indicated that the disassembly of M2 dimer by the K95E mutation almost eliminated the enzyme activity in vitro and in cells (Fig. 2, C and E). Whereas the K95Q mutant partially recovered the dimerization and the enzyme activity in comparison with the K95E mutant, the K95E/E98K double mutations that maintain the charge balance at the Lys-95 cluster completely recovered the dimer form and enzyme activity (Fig. 3). The evidence demonstrated that the Lys-95 charged residue cluster functions as critical salt bridges for the dimer assembly, and the dimer form of M2 is essential for human RR activity.

It was suggested based on structural analyses over past 20 years that E. coli R2 dimer interaction is formed by the first layer of helices, the first β-sheet, and the random-coil structure of the N-terminal residues (45); the subunit interface of mouse R2 is held together mainly by hydrophobic interactions, such as with Phe-102, Phe-165, and Ala-168, and charged interactions may also occur in the dimer interface, such as with Lys-96 interacting with Glu-106, Glu-175, and Glu-99, and Arg-160 with Glu-144 and Glu-148 (48); in addition, the interaction of Arg-80 with Glu-144 and also that of Arg-79 with Glu-192 are possibly involved in the interface interaction (49). However, there was no further biological evidence to support these predictions.

The eukaryotic R2s share about 60–82% sequence identity, whereas it is 25% between E. coli and mouse R2 (48). Our study demonstrated that among the three clusters of charged residues at the dimer interface of human M2 (Fig. 1E), the Lys-95 cluster (Lys-951/Glu-981/Glu-1052/Glu-1742), corresponding to Lys-96/Glu-106/Glu-175/Glu-99 in mouse R2 (50), was well structure-supervised with the small subunits of other representative class Ia RRs (Fig. 4). The in vitro and in vivo biological analyses revealed that the charged interaction of the Lys-95 cluster was critical to M2 dimer formation (Figs. 2 and 3). The conservation suggests a common function of the Lys-95 charged residue cluster in class Ia RR small subunits. We also examined the contribution of the other two clusters (Arg-781/Glu-1912 and Arg-791/Glu-1472/Arg-1591), which partially correspond to the predicted residues in mouse R2 (49, 50), to the dimerization and activity of M2. However, co-immunoprecipitation and RR activity assays showed a less important role of the two clusters for the dimerization (Fig. 2, A and C).

Our computational alanine scanning predicted that the interfacial hydrophobic residues, such as Phe-80, Phe-83, and Phe-101 (corresponding to Phe-81, Phe-84, and Phe-102 in mouse R2), might be involved in human M2 dimer assembly (Fig. 1D). The size exclusion experiments and RR assays showed that Phe-83 and Phe-101 had a certain influence on M2 dimer formation and enzyme activity (Fig. 2, A and D). Although the F80A mutation significantly reduced the RR activity, it did not affect the dimerization of M2, as shown in co-immunoprecipitation and size exclusion chromatography experiments (Fig. 2, A, C, and D). Multiple mechanisms are involved in the regulation of RR activity in mammalian cells (51). The critical role and related mechanism of Phe-80 for human M2 activity are worth studying in the future. Thus, all of the experimental evidence suggested that the K95E charged residue cluster plays a comparatively more important role in human M2 dimer formation.

There are two primary possibilities that the dimer form of M2 is essential for the enzyme activity of human RR. The first possibility is that the dissociation of M2 dimer by the K95E
mutation may affect the generation or stability of the activity-essential diiron-tyrosyl radical cofactor in M2. However, the measurements of the radical and iron contents showed that the K95E mutant housed the diiron and tyrosyl radical cofactor similarly to the wild-type M2 (Fig. 5 and Table 4), indicating that the disruption of the dimer interaction by the K95E mutation has no significant impact on the activity-essential cofactor in M2. The second possibility is that the disassembly of M2 dimer may interfere with its interaction with M1 because the dimer conformation is probably required for M1 binding and the PCET between the two subunits. This hypothesis was supported by the co-immunoprecipitation investigations and competitive enzyme inhibition assays (Fig. 6, A and B). The above evidence suggested that although the monomer was capable of generating the activity-essential cofactor similar to the dimer, the conformation of the dimer form was needed for M1 binding to constitute a functional human RR. In the future, the structure determination of an active holo-class Ia RR will help to further clarify this mechanism.

The RR small subunit of S. cerevisiae is a ββ' heterodimer. β' is structurally homologous to β but lacks important ligating side chains and contains no metallocofactor. Although β' is catalytically inactive, it is required for converting β into a conformation that is competent for iron loading and Y' formation by forming a stable 1:1 ββ' complex in vitro and in vivo (16, 17, 46). Human RR M2 is a homodimer. We showed that the generation and stability of the diiron-tyrosyl radical cofactor was not dependent on the dimer form; however, a monomer probably lacks the quaternary structure for binding and communicating with M1.

The reduction of ribonucleotides is the rate-limiting step of DNA synthesis. Cell cycle-regulated R2 is responsible for providing dNTPs in actively dividing cells, and the DNA damage-inducible p53R2 is required for replenishing dNTP pools in cells under genotoxic stress (50, 52). Because of its critical function in DNA replication and repair, RR is tightly regulated in cells by multiple mechanisms, including subcellular translocation. There are two opinions about the intracellular location of dNTP synthesis in mammalian cells. In early studies, R1 and R2 were observed to predominantly localize to the cytoplasm. Therefore, ribonucleotide reduction was thought to take place in the cytoplasm, and the dNTPs were presumed to be transported into the nucleus for DNA synthesis (53). Recently, the “replitase” model has suggested that NDPs, rather than free dNTPs, are “channeled” directly into DNA synthesis in the nucleus. It is argued that RR subunits translocate into the

![Figure 7](http://www.jbc.org/)

**Figure 7.** HeLa cells were transfected with the specific siRNA against human RR M2 gene (siM2) or the scramble siRNA (siCON) as control. The construct encoding the wide type or K95E mutant M2 protein or the empty vector control (EV) was co-transfected with siM2 or siCON, respectively. The cells were harvested for cell cycle analysis by flow cytometry (A) and for the cell proliferation assay using the CCK-8 cell-counting kit (in triplicate; * and #, p < 0.05, t test) (B, C). The expression of the wild type and mutant M2 proteins in the transfected cells were measured by the immunoblotting method. Error bars, S.D.
nucleus individually and assemble there to form the active holoenzyme (54). In response to DNA damage, human M2 and p53R2 move rapidly from the cytoplasm into the nucleus to form an active complex to ensure the local availability of dNTPs at DNA damage sites for repair (37). However, the translocation mechanism of mammalian RR subunits in cells is still unclear. In S. cerevisiae, three mechanisms are involved in the distribution and translocation of R2: Dif1-dependent nuclear import, retention by the nuclear anchor Wtm1, and Cnm1-mediated nuclear export (55, 56). However, no similar anchoring or transporting mechanisms have been reported for R2 in mammalian cells so far. Our immunofluorescent and EYFP fusion protein–fluorescent microscopy analyses showed that whereas the wild-type M2 mainly located in the cytoplasm, the K95E mutant changed its distribution to both the cytoplasm and nucleus (Fig. 6, B and C). The disordered distribution due to M2 dimer disassembly may disturb the exertion of RR function in cells. It is possible that only the dimer form of M2 possesses an appropriate conformation for passing through the nuclear pore or can interact properly with certain proteins similar to that in S. cerevisiae for normal distribution in cells, which deserves further investigation.

RR is a critical enzyme for cell fate. Increased expression and activity of human RR has been associated with malignant transformation and cancer development, which makes it an important target for anticancer agents (57) RR inhibitors, such as the small compounds hydroxyurea, triapine, and gemcitabine; the antisense oligonucleotides, such as GTI-2040; and some oligopeptides inhibiting R1–R2 polymerization have been used in clinical cancer treatment or are being vigorously investigated (58) Our study demonstrated that disruption of M2 dimerization extinguished the enzyme activity of human RR, suggesting that disruption of M2 dimerization is a potential strategy for RR inhibition.

In summary, this study demonstrated for the first time that the conserved Lys-95 charged residue cluster plays a predominant role in M2 dimerization, which is essential for human RR activity in vitro and in vivo. Disassembly of the M2 dimer affects its interaction with the large subunit and its distribution in cells, ultimately leading to abnormality of the function of human RR. These new findings extend our understanding of the structural architecture and functional mechanism of the multisubunit enzyme RR and may propose a potential site for the design of novel RR inhibitors.

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The Conserved Lys-95 Charged Residue Cluster Is Critical for the Homodimerization and Enzyme Activity of Human Ribonucleotide Reductase Small Subunit M2

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