A Mutational Epitope for Cytochrome c Binding to the Apoptosis Protease Activation Factor-1*

Received for publication, October 26, 2000, and in revised form, December 6, 2000
Published, JBC Papers in Press, December 8, 2000, DOI 10.1074/jbc.M009773200

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Cytochrome c (Cc) binding to apoptosis protease activation factor-1 (Apaf-1) is a critical activation step in the execution phase of apoptosis. Here we report studies that help define the Cc:Apaf-1 binding surface. It is shown that a large number of Cc residues, including residues 7, 25, 39, 62–65, and 72, are involved in the Cc:Apaf-1 interaction. Mutation of residue 72 eliminated Cc activity whereas mutations of residues 7, 25, 39, and 62–65 showed reduced activity in an additive fashion. The implications of this binding model for both recognition and modulation of protein-protein interactions are briefly discussed.

The importance of apoptosis, or “programmed cell death,” for multicellular organisms is well established. One key apoptotic pathway in vertebrates is triggered when the mitochondrial protein cytochrome c (Cc)1 is released from mitochondria (1). In the cytosol, Cc binds strongly to apoptosis protease activation factor-1 (Apaf-1) (2). In the presence of dATP and perhaps other cofactors, the Cc:Apaf-1 complex assembles into a multimeric “apoptosome” that binds and activates a protease zymogen, procaspase-9 (3–6). The activated caspase-9 cleaves and activates procaspase-3 and other downstream caspases in a “caspase cascade”. The active caspases in turn cleave many intracellular substrates, thereby disabling important cellular processes and breaking down structural components of the cell (see Refs. 7 and 8 and for reviews see Refs. 9–12). The initial Cc:Apaf-1 binding is a critical step in this process and therefore represents a potential therapeutic target. To date, our understanding of this interaction is very limited. Here we report studies that help identify the molecular determinants of this interaction.

Horse Cc can initiate caspase activation in an in vitro reconstitution system containing Cc, Apaf-1, dATP, procaspase-9, and procaspase-3. However, the highly homologous yeast Cc cannot substitute horse Cc to initiate activation (13) and, in fact, does not measurably bind to Apaf-1 (as described under “Results and Discussion”). This result is surprising, because the sequences of yeast Cc and horse Cc are highly homologous. These sequences are most strongly conserved at the “front face lysine patch,” which is involved in all previously studied physiological complexes of Cc (14) (Fig. 1, A and B). For example, yeast cytochrome c peroxidase can bind almost equally well with yeast and horse Cc (15).

Our current studies focus on key differences between the horse and yeast Cc, including residues 1–9, 53–60, 62–65, 25, and 39, as well as position 72, which is trimethylated in fungi but not in higher eukaryotes (the horse cytochrome c numbering system is used in this article). Two complementary classes of mutagenesis experiments were carried out, and the mutant Cc were used to activate procaspase-9 in the reconstituted Apaf-1-mediated caspase activation reaction. In the first set of experiments, horse Cc residues were mutated to the yeast sequence, whereas in the complementary experiments, yeast Cc residues were replaced by the horse Cc sequence.

EXPERIMENTAL PROCEDURES

Fluorescence Polarization Methods—Steady-state fluorescence experiments were performed using a Photon Technologies International (South Brunswick, NJ) Quantamaster fluorometer equipped with a PowerArc xenon arc lamp excitation source. The instrument was fitted with Glan-Thompson manual polarizers. Data were recorded using Felix software for Windows, Version 3.1. Polarization experiments were performed in a 10-mm pathlength quartz cuvette containing 2 ml of Buffer A (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM Na2EDTA, pH 7.5) as described previously (16). Apaf-1 (in the presence or absence of excess Fe yeast Cc) in Buffer A (2–4 µM) was titrated into a 100 mM solution of yeast porphyrin Cc, fluorescent derivative of yeast Cc where the free base porphyrin is used in place of the iron porphyrin (porYCc) or fluorescent derivative of horse heart Cc with zinc substituted for iron in the heme (ZnHCc) in the presence of 25 µM dATP. PorYCc was excited at 515 nm, and emission was monitored at 620 nm. ZnHCc was excited at 550 nm, and emission was monitored at 587 nm (16).

Horse Cc Variants Generated in Escherichia coli—The horse Cc mutants (from I to XXI in Table I) were also generated based on plasmid pBTR1 (18) by replacing the horse sequence with the corresponding yeast sequence of yeast. An E. coli/pBTR1 cloning protocol was used to generate all the variants. All the mutants were cloned into XL-1 Blue to generate the lysine 72 trimethylated horse Cc variant XXII. CNBr digests and 1-D NMR experiments confirm the existence of the trimethyl group at position 72.

Yeast Cc Variants Generated in Yeast—Yeast strain B-8581 was used to generate the lysine 72 trimethylated horse Cc variant XXII. CNBr digests and 1-D NMR experiments confirm the existence of the trimethyl group at position 72.

Yeast Cc Variants Generated in Yeast—In vivo transformation method was used to mutate the yeast amino acids 1–9 and 53–60 (17). The parental yeast strain B-8163, which contains a frameshift/stop codon at position 6 (TAAGT) was used to generate mutant XXVI (S22, A3V, K4E, A7K, T8K, L91). Similarly, yeast strain B-8162, which contains a frameshift/stop codon at position 52, is used to obtain mutant XXVII (L37K, K4N, N56G, V571, L587T, D60K).

Yeast Cc Variants Generated in E. coli—Plasmid pBTR1 (18) (a

* This work is supported in part by National Institutes of Health Grants GM59348 (to G. M.) and GM57158 and Welch Foundation Grant 11412 (to X. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: Cc, cytochrome c; Apaf-1, apoptosis protease activation factor-1; porYCc, porphyrin yeast cytochrome c; ZnHCc, zinc derivative of horse cytochrome c.
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FIG. 1. A, sequence alignment for human, horse, Drosophila, and yeast cytochromes c (26). The horse cytochrome c numbering system is used. A single letter code X is used to represent the e-N-trimethyl lysine residue. Identical amino acid residues are labeled by boxes with gray shading. B, the helix-and-strand structures of yeast Cc (1YCC) and horse Cc (1HRC) are overlaid in three dimensions. This figure was generated by Raster3D (27) and MOLSCRIPT (28). The yeast Cc is red with heme in cyan, whereas horse Cc is purple with heme in blue.

generous gift from Dr. Grant Mauk) was used to create nontrimethylated yeast Cc at position 72 (variant XXIII). The N-terminal codon was changed from Thr to Ala. A C102T mutation was introduced to diminish potential dimerization through disulfide bonds. Based on this plasmid, site-directed mutagenesis method is used to generate mutants XXIV and XXV.

Isolation of Recombinant Cytochrome c Mutants—Cytochrome c variants expressed in E. coli were purified by a slightly modified procedure of Hampsey et al. (19) and Cutter et al. (20). Cell pellets from 2-liter culture were resuspended in lysis buffer (50 mM Tris, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 7.5, 10 mM MgCl₂, and 20 μM/ml DNase I). The lysate was treated with a French press (10,000 p.s.i.) and centrifuged at 40,000 × g for 30 min. The crude protein was cut at 55% (NH₄)₂SO₄ (326 g/liter at 4 °C), and the supernatant was dialyzed against 12.5 mM potassium phosphate buffer, 1 mM EDTA, 2 mM 2-mercaptoethanol, pH 7.2. Ion-exchange purification (CM-Sepharose; Sigma) followed by gel filtration (Superdex 30; Amersham Pharmacia Biotech) gave cytochrome c with a purity index A₂₈₀/A₅₅₀ of about 4.5. Homogeneity and purity of the variants were confirmed by electrospray mass spectrometry and SDS polyacrylamide gel electrophoresis. Conformationally sensitive bands in the variants are essentially conserved, suggesting that the Cc variants are folded properly, and the structures are intact. It should be noted that, unlike wild type horse Cc, the N-terminal glycine is not acetylated in horse Cc expressed in E. coli.

Procaspase-3 Assay of Caspase-9 Activation (2)—2 μl of Apaf-1 (100 ng) was incubated at 30 °C for 1 h with 1 μM dATP, 2 μM purified procaspase-9 (12.5 ng), in vitro-translated 35S-labeled procaspase-3, and varying concentrations of cytochrome c variants. The samples were subjected to 15% SDS polyacrylamide gel electrophoresis and then transferred to a nitrocellulose filter, which was subsequently exposed to a phosphorimaging plate and visualized in a Fuji BAS-1000 phosphorimager.

RESULTS AND DISCUSSION

Fluorescence Polarization Data Show That Yeast Cytochrome c Binds to Apaf-1 Much More Weakly than Does Horse Cytochrome c—ZnHCc and porYCc were used as fluorescent probes because of their high structural homology to nonfluorescent iron cytochrome c (21). When Apaf-1 (molecular mass = 130 kDa) was titrated into ZnHCc (molecular mass = 12 kDa) in the presence of micromolar dATP, the fluorescence polarization increased dramatically. Under the same experimental conditions, when Apaf-1 plus a 20-fold excess of yeast Cc was titrated into a ZnHCc solution, a similar trend of fluorescence polarization increase was observed (Fig. 2A). This implies that yeast Cc does not compete with ZnHCc for binding Apaf-1. In a direct titration experiment, no fluorescence polarization change was observed, indicating that the yeast Cc did not compete with the horse Cc for Apaf-1 binding.

FIG. 2. Polarization titrations of yeast and horse heart derivatives of Cc with Apaf-1 in the presence of 50 μM dATP. A, +, ZnHCc (150 nM) titrated with Apaf-1; ○, Apaf-1 was mixed with a 20-fold excess of yeast FeCc, and the mixture was titrated into a solution of 150 nM ZnHCc. If the yeast cytochrome c interacted with the Apaf-1, significantly less binding should have been observed when the Apaf-1/yeast Cc mixture was added to the ZnHCc. However, the binding of ZnHCc with Apaf-1 under both sets of conditions looked very similar, indicating that the yeast Cc did not compete with the horse Cc for Apaf-1 binding, P/ Po, measured polarization ratio. B, PorYCc (150 nM) was titrated with Apaf-1 (ex = 515 nm, em = 620 nm), and polarization of the PorYCc remained constant at approximately P = 0.16, indicating that no binding had occurred. Upon addition of ZnHCc to the yeast PorCc/Apaf-1 mixture, polarization of the ZnHCc (ex = 550 nm, em = 587 nm) increased significantly, indicating that the horse, but not yeast, Cc bound to Apaf-1.
FIG. 3. **Procaspase-3 cleavage assay of caspase-9 activation** (2) induced by cytochrome c variants. Aliquots of 2 μl of Apaf-1 (100 ng) were incubated at 30 °C for 1 h with 1 mM dATP, aliquots of 2 μl of purified procaspase-9 (12.5 ng), in vitro-translated 35S-labeled procaspase-3, and various concentrations of cytochrome c variants as indicated. The samples were subjected to 15% SDS polyacrylamide gel electrophoresis and then transferred to a nitrocellulose filter, which was subsequently exposed to a phosphorimaging plate and visualized in a Fuji BAS-1000 phosphorimager. Reference Table I for the names of Cc variants.

**TABLE I**

*Information about the variants discussed in this paper*

All the proteins are purified to homogeneity as indicated under “Experimental Procedures.”

| Name   | Variant type                                      |
|--------|-------------------------------------------------|
| I      | K7E (horse Cc background)                        |
| II     | K8E (horse Cc background)                        |
| III    | K7E, K8E, V11K (horse Cc background)             |
| IV     | K7Q, K8Q (horse Cc background)                   |
| V      | K7E, K8E (horse Cc background)                   |
| VI     | K7A (horse Cc background)                        |
| VII    | K39H (horse Cc background)                       |
| VIII   | K25P, K39H (horse Cc background)                 |
| IX     | E62N (horse Cc background)                       |
| X      | K7E, K8E, E62N (horse Cc background)             |
| XI     | K7E, K8E, E62N, K39H (horse Cc background)       |
| XII    | K7E, K8E, E62N, K25P, K39H (horse Cc background) |
| XIII   | K7A, E62N, K25P (horse Cc background)             |
| XIV    | K7A, E62N, K39H (horse Cc background)             |
| XV     | K7A, E62N, K25P, K39H (horse Cc background)      |
| XVI    | E62N, T63N, L64M, M65S (horse Cc background)     |
| XVII   | K7E, K8E, E62N, T63N, L64M, M65S (horse Cc background) |
| XVIII  | K7E, K8E, E62N, T63N, L64M, M65S, K25P (horse Cc background) |
| XIX    | K7E, K8E, E62N, T63N, L64M, M65S, K39H (horse Cc background) |
| XX     | K7E, K8E, E62N, T63N, L64M, M65S, K25P (horse Cc background) |
| XXI    | K72A (horse Cc background)                       |
| XXII   | Trimethylated Lys-72 (horse Cc background)        |
| XXIII  | Nontrimethylated Lys-72, T5A, C102T (yeast Cc background) |
| XXIV   | Nontrimethylated Lys-72, T5A, C102T, A7K, P25K, H39K, N62E, N63T, M64L, S65M (yeast Cc background) |
| XXV    | Nontrimethylated Lys-72, C102T, A7K, P25K, H39K, N62E, N63T, M64L, S65M, −5 to −1 deletion (yeast Cc background) |
| XXVI   | S2D, A3V, K4E, A7K, T5A, L64M, M65S (yeast Cc background) |
| XXVII  | S2D, A3V, K4E, A7K, T5A, L64M, M65S, K25P (yeast Cc background) |

![A] Procaspase-3 cleavage assay of caspase-9 activation (2).

**B**

![B] Procaspase-3 cleavage assay of caspase-9 activation (2).

**C**

![C] Procaspase-3 cleavage assay of caspase-9 activation (2).
observed when the fluorescent yeast protein porYCc was titrated with Apaf-1 (Fig. 2B). From these two sets of experiments, we conclude that yeast Cc has relatively weak binding affinity for Apaf-1 compared with horse Cc.

**Site-directed Mutagenesis Results Indicate That a Large Part of the Cc Surface Residues Are Involved in Cc:Apaf-1 Recognition**—All the variants we examined are summarized in Table I. Here we found that when positions 7, 25, 39, 62–65, or 72 of the active horse sequence were mutated to the corresponding residues of inactive yeast Cc sequence, the induced caspase-9 activity in an *in vitro* reconstitution procaspase-3 cleavage assay was diminished. The effects of replacements seemed synergistic when the double and triple mutants were examined. By contrast, replacing residues 1–9 (XXVI) or 53–60 (XXVII) in the yeast background did not provide sufficient activity to be detected by our assay (data not shown).

When yeast sequence variations were introduced into horse Cc, several mutated Cc showed significant effects on caspase-9 activation. Replacement of residues 7 and 8 (variant IV) resulted in a 10-fold decrease in the procaspase-3 cleavage assay when compared with wild type horse Cc (Fig. 3A, lane 3). The variant VII (K39H) produced an ~5-fold decrease in activity. The variant VIII (K25P/K39H) generated a more than 10-fold drop in activity (data not shown). Replacement of residues 62–65 (variant XVI) decreased the activity about 10-fold with the major effect contributed by 62 (variant IX, Fig. 3A, lane 4). This effect is amplified by introducing multiple mutations. For example, the variants XI (K7E/K8E/E62N/K39H) (Fig. 3B, lane 6) and XVIII (K7E/K8E/E62N/T63N/L64M/M65S/K25P) (Fig. 3A, lane 5) showed more than 1000-fold decrease in the caspase activation activity.

Interestingly, horse Cc, when expressed and purified from yeast, is trimethylated at position 72. This variant, XXII (K72X), had little caspase-9-activating activity (Fig. 3B, lane 8). Similar results have been reported previously (24). The importance of this position was further underscored by the variant XXI (K72A), which showed no detectable caspase-9-activating activity at all (Fig. 3B, lane 7). We also generated yeast Cc from *E. coli*. The Lys-72 of recombinant yeast Cc generated this way is not trimethylated (18). This variant, XXIII, showed significant caspase-9-activation activity, even though the activity was well below the wild type horse Cc (Fig. 3C, lane 2).

When the XXIII variant was further modified from the yeast sequence to the horse sequence at the positions 7, 25, 39, and 62–65 (variant XXIV), the caspase-9 activation was enhanced to a level similar to the wild type horse Cc (Fig. 3C, lane 9). The activity of the variant XXIV was further enhanced when the N-terminal sequence (~5 to ~1) found in fungi was eliminated. The resulting variant XXV showed apoptotic activity that was equal to or that even exceeded that of wild type horse Cc (Fig. 3C, lane 4).

In summary, these results showed that when residues 7, 25, 39, and 62–65 of yeast were substituted with the corresponding horse Cc amino acids, caspase-9 activation was dramatically increased. On the other hand, when these same amino acids in horse Cc were replaced by the corresponding yeast Cc sequence, a more than 1000-fold drop in caspase-9 activation was observed. Taken together, these results indicate that 7, 25, 39, and 62–65 are critical amino acids for Cc:Apaf-1 interaction.

Because positions 7, 25, 39, 62–65, and 72 are so widely dispersed on the surface of Cc (Fig. 4), we conclude that Cc:Apaf-1 recognition involves a large region of the Cc surface. It seems that Cc is almost fully “wrapped” by Apaf-1, which is consistent with the high binding constant of Cc and Apaf-1 (8). The hypothetical binding model for Cc binding to Apaf-1 includes the Apaf-1 WD-40 domain (22, 23), a beta-sheet barrel that could encompass much of the Cc surface. Although the binding surface is extensive, the amino acids on the binding interface do not seem to contribute equally. In this work, we have pinpointed several central amino acids that substantially affect the apoptotic activity of Cc when mutated. In contrast, mutations at other amino acids did not affect its apoptotic activity. For example, replacement of lysine 7 had a much reduced apoptotic activity than the replacement of lysine 8, and replacement of valine 11 had no effect at all (data not shown).

The contribution of individual residues is almost additive. The variants V (K7E/K8E), IX (E62N), and VIII (K25P/K39H) individually showed an ~10-fold reduction in caspase-9-activating activity. When variant V and variant IX were combined to make the variant X (Fig. 3B, lane 2), a 100-fold drop in activity was observed. The variant XII, which combines all three mutations, exhibited a greater than 1000-fold drop in caspase-9 activation (Fig. 3A, lane 7). The similar synergistic trend was seen with the variants XVI (E62N/T63N/L64M/M65S) (data not shown), XVII (data not shown), XVIII, XIX, and XX (Fig. 3A, lanes 5, 6, and 8), variants VI (K7A) (data not shown), XII, XIV, and XV (Fig. 3B, lanes 3, 4, and 5).

**Conclusions**—The binding interface inferred for Cc:Apaf-1 binding is unusual. All previous (electron transfer) partners of Cc bind at the “front face” of Cc, with the lysine-rich interface in close proximity to the heme (6). In contrast, in Apaf-1 binding, Cc uses not only this front face, but also an opposite surface centered around positions 39 and 62–65. This large interface explains both the unusually high binding affinity between Cc and Apaf-1 (16) and the ionic strength dependence of the binding (25). The binding region identified here also explains the striking differences in activity between the highly homologous horse Cc and yeast Cc (13). Interactions from both the 62–65 region and specific front face lysine (*e.g.* 7, 25, and 72) contribute to overall binding and activity. Because the contributions from each subset appear to be essentially independent, a single subset might be targeted to affect overall binding. We are investigating a possibility to use small molecules to affect the binding of Cc to Apaf-1. Initial results suggest that designed synthetic molecules can indeed compete with the Cc:Apaf-1 binding interaction.

**Acknowledgments**—T. Y. thanks Yudong Wu, Drs. Stacy S. Springs, Martin A. Case, Rachael A. Kipp, and Federico Rosell for helpful discussions and critical reading of manuscript, and Dr. Chunying Du for providing the reagents and making the figures.
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