*Apoptosis* 2005; **10**: 797–808


# Comparative analysis of proapoptotic activity of cytochrome *c* mutants in living cells

**G. V. Sharonov, A. V. Feofanov, O. V. Bocharova, M. V. Astapova, V. I. Dedukhova, B. V. Chernyak, D. A. Dolgikh, A. S. Arseniev, V. P. Skulachev and M. P. Kirpichnikov**

Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117997, Moscow, Russia (G. V. Sharonov, A. V. Feofanov, O. V. Bocharova, M. V. Astapova, D. A. Dolgikh, A. S. Arseniev, M. P. Kirpichnikov); A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, 119899, Russia (V. I. Dedukhova, B. V. Chernyak, V. P. Skulachev)

**A non-traumatic electroporation procedure was developed to load exogenous cytochrome *c* into the cytoplasm and to study the apoptotic effect of cytochrome *c*, its K72-substitued mutants and "yeast → horse" hybrid cytochrome *c* in living WEHI-3 cells. The minimum apoptosis-activating intracellular concentration of horse heart cytochrome *c* was estimated to be $2.7 \pm 0.5 \mu$M ($47 \pm 9$ fg/cell). The equieffective concentrations of the K72A-, K72E- and K72L-substituted mutants of cytochrome *c* were five-, 15- and 70-fold higher. The "yeast → horse" hybrid created by introducing S2D, K4E, A7K, T8K, and K11V substitutions (horse protein numbering) and deleting five N-terminal residues in yeast cytochrome *c* did not evoke apoptotic activity in mammalian cells. The apoptotic function of cytochrome *c* was abolished by the K72W substitution. The K72W-substituted cytochrome *c* possesses reduced affinity to the apoptotic protease activating factor-1 (Apaf-1) and forms an inactive complex. This mutant is competent as a respiratory-chain electron carrier and well suited for knock-in studies of cytochrome *c*-mediated apoptosis.**

*Keywords:* apoptosis; confocal spectral imaging; cytochrome *c*; electroporation; fluorescence microscopy; mutagenesis.

## Introduction

Cytochrome *c* activates programmed cells death, apoptosis, when mitochondria release it to the cytosol.[1,2] Evidently, many biological, biotechnological and medical tasks can be solved by means of delicate adjustment of the cell death program. To this end, understanding the factors and mechanisms of caspase activation and activity is required. It was shown that the apoptotic cascade can be activated *in vitro* by the addition of cytochrome *c* and dATP to the cytosol from nonapoptotic cells.[1,3,4] Studies of structural determinants of cytochrome *c* proapoptotic activity revealed a set of functionally important amino acid residues. The residues 7, 25, 39, 62–65 and 72 were reported to be involved in the interaction of cytochrome *c* with Apaf-1.[5] Horse heart cytochrome *c* with trimethylated Lys-72 residue manifested low caspase-9 activating ability in an *in vitro* reconstitution system containing cytochrome *c*, Apaf-1, dATP, procaspase-9 and procaspase-3[5] as well as in Xenopus egg and mammalian extracts.[6] Site-directed mutations also confirmed the importance of Lys-72 for apoptotic function of cytochrome *c* in Xenopus egg and mammalian extracts.[7] Interestingly, the introduction of mutations at the position 72 did not affect the electron transfer and antioxidant properties of cytochrome *c*.[6,7] This fact offers an intriguing possibility of a single residue mutation being able to abolish selectively the pro-apoptotic function but not the electron transfer and antioxidant activities of cytochrome *c*.

Besides the apoptotic systems reconstituted in solution, there is a complementary approach for investigation of the cytochrome *c* induced apoptotic pathway in living cells. It is based on the introduction of exogenous cytochrome *c* into the living cell cytoplasm. This approach is of great importance both to verify to what degree the reactions observed *in vitro* are equivalent to the intracellular processes and to study the pro-apoptotic role of cytochrome *c*, its partners and apoptosis-regulating factors *in situ*.[8–13] Currently, three techniques are exploited to load cytochrome *c* into the cytoplasm of cells: microinjection technique,[8,14–18] electroporation procedure[9–12] and the method of pinocytic loading of macromolecules.[13] Independently of the way of cytochrome *c* loading, the investigators agree that exogeneous cytochrome *c* induces activation of the caspase cascade in living cells and, finally, produces other apoptotic effects such as cell shrinkage, chromatin condensation, oligonucleosomal fragmentation of DNA, fragmentation of nuclei and phosphatidylserine externalization.

Correspondence to: A. V. Feofanov, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117997, Moscow, Russia. Tel.: +7-095-3366455; Fax: +7-095-3361766; e-mail: alexei@nmr.ru

It should be noted that a microinjection technique is suitable for adherent cells and treats a restricted number of cells only. As an advantage, investigation of cellular response can be initiated immediately after the microinjection.[15] The technique is hardly applicable to cells in suspension, when injected cells should be further treated with other agents and/or followed for a long time. The electroporation procedure and pinocytic loading works well with cells in suspension and treats millions of cells simultaneously. Microinjected cells are the subject of investigation with optical microscopy, whereas not only microscopy, but also flow cytometry and biochemical analyses of cellular extracts are applicable to electroporated and pinocytically-loaded cells.

A statement that pinocytic loading of cytochrome *c* in cells is a "relatively gentle technique" as compared with "the disruptive techniques of microinjection or electroporation"[13] should be considered as a literary hyperbole (exaggeration), since this method is based on the enhancement of pinocytosis at the hyper-osmolar conditions followed by lysis (disruption) of intracellular vesicles, pinosomes, induced by hypo-osmolar shock. As for the viability of cells, here we report on an optimized electroporation procedure that results in a specific activation of apoptosis in up to 80% of cells after cytochrome *c* electroinjection and in a very low level of dead and apoptotic cells in control experiments.

We show that the optimized electroporation procedure allows one to compare relative pro-apoptotic activities of mutants of cytochrome *c* in living cells. We have studied four K72-substituted mutants (K72A, K72E, K72L and K72W) of horse heart cytochrome*c* (hereafter referred as h(K72A), h(K72E), h(K72L) and h(K72W), respectively) and "yeast → horse" hybrid cytochrome *c* in the living WEHI-3 cells.

High sensitivity of our *in situ* apoptosis system combined with the ability of the confocal spectral imaging (CSI) technique to resolve multiple fluorescence signals and quantify fluorophore concentrations[19–22] allowed us to characterize particular aspects of cytochrome *c*-induced apoptosis in WEHI-3 cells.

## Materials and methods

### Chemicals and solutions

Cytochrome *c* from horse heart, cytochrome *c* (iso-1) from *Saccharomyces cerevisiae*, cyclosporin A, tetramethylrhodamine (TR) isothiocyanate, rhodamine 123 (Rh), Hoechst33342 and propidium iodide (PI) were supplied by Sigma (St. Lois, MO, USA). Recombinant human annexin V conjugated with R-phycoerythrin (AnV) was purchased from Caltag Laboratories (Burlingame, CA, USA). The other chemicals used in this work were of analytical-reagent grade.

According to the manufacturer (Eppendorf AG, Hamburg, Germany) specification the hypoosmolar electroporation medium contained 25 mM KCl, 0.3 mM $KH_2PO_4$, 0.85 mM $K_2HPO_4$ and myo-inositol providing 90 mOsmol/kg (pH $= 7.2 \pm 0.1$). Conductivity of the medium at 25°C was $3.5 \pm 0.4$ mS/cm.

### Conjugation of cytochrome *c* with TR isothiocyanate

The solution of TR isothiocyanate in DMSO (20 mg/ml, 40 $\mu$l) was slowly added to the solution of cytochrome *c* (2 mg/ml, 400 $\mu$l) in sodium bicarbonate buffer (100 mM, pH 8.5) at continuous stirring. Reaction mixture was incubated during 12 h at 4°C. The reaction products were separated using 100 mm column filled with fine grade Sephadex G-50 (Amersham Biosciences AB, Uppsala, Sweden) after equilibration with phosphate buffered saline (PBS). Elution was performed with PBS at 0.6 ml/min. The degree of labeling of the collected fractions was estimated by measuring the absorbance of cytochrome *c* at 410 nm (extinction coefficient of 80000 $M^{-1}$ $cm^{-1}$) and TR at 555 nm (extinction coefficient of 65000 $M^{-1}cm^{-1}$) after correction for the contribution of cytochrome *c* absorption band at 523 nm. The fraction having the TR/cytochrome *c* molar ratio of 0.9 was desalted and concentrated by double centrifugation of solution through the Ultrafree-4 centrifugal filter unit with Biomax membrane having the nominal molecular weight limit of 10000 (Millipore, Bedford, MA, USA). This fraction was mixed with native cytochrome *c* to produce final TR/cytochrome *c* molar ratio of 0.3, and the resulted solution (hereafter referred as TR-cytochrome *c*) was used for the electroporation experiments.

### Cytochrome *c* mutants

The K72A, K72E, K72L and K72W recombinant mutants of horse heart cytochrome *c* were expressed with substitutions at the Lys-72 position as described previously.[7,23] "Yeast → horse" hybrid was created by introducing S2D, K4E, A7K, T8K, and K11V substitutions (horse protein numbering) and deleting five N-terminal aminoacid residues in yeast cytochrome *c*.[7] The final purity of the proteins was ~95% according to SDS/PAGE. The stock solutions of cytochrome *c* and its mutants were prepared in the hypoosmolar electroporation medium at 1 mM concentration.

### Cells

Murine monocytic WEHI-3 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated

fetal calf serum (FCS) and 2 mM L-glutamine (complete medium) at 37°C in humidified air with 5% (v/v) $CO_2$. Cells were subcultured two times per week. Seeding density was $2 \times 10^5$ cells per ml.

## Electroporation procedure

For electroporation experiments, exponentially growing cells (two days of growth) were harvested and washed with RPMI 1640 medium at room temperature. Hypoosmolar electroporation medium (0.85 ml, 22°C) was added to cell precipitate ($2 \times 10^6$ cells per ml) with or without cytochrome *c*, and cells were electroporated after 5 min pre-incubation in the medium. Eight minutes after the pulsing, cells were gently transferred to a 4-ml centrifuge tube, precipitated with centrifugation, and the electroporation medium was substituted with the complete medium (3 ml). Cells were then incubated directly in the centrifuge tube (37°C, 5% $CO_2$) for 0.5–4 h depending on the task. Total time of cell exposure to the electroporation medium did not exceed 17 min. According to our experience, a postelectroporation substitution of the electroporation medium with the complete medium eliminated noticeable toxic effect, which was observed, if cells were incubated in the electroporation medium (0.8 ml) supplemented with the complete medium (5 ml). Centrifugation of cells was performed at 175 × g for 3 min. Electroporation of cells was carried out with Multiporator (Eppendorf AG, Hamburg, Germany) in disposable sterile electroporation cuvettes (0.8 ml) with the 4 mm gap between embedded aluminum electrodes (Eppendorf AG). Cell suspension was subjected to a single exponentially decaying electric pulse with the 1900 V/cm electric field strength. The pulse length (defined as time for $e$-fold decrease of the pulse amplitude, where $e$ is the base of the natural logarithm) was 90 $\mu$s.

## Apoptosis assay

Two delays between electroporation of cells and their examination under microscope were usually used, namely, 110 and 140 min. Cells (1.5 ml, $\sim 6 \times 10^5$ cells per ml) were placed on ice for 10 min, washed once with the ice-cold AnV-binding medium (140 mM NaCl, 2.5 mM $CaCl_2$, 10 mM HEPES/NaOH, pH 7.4) and resuspended in 200 $\mu$l of the AnV-binding medium (22°C). Stock solution of AnV (2.5 $\mu$l), Hoechst33342 (4 $\mu$M) and PI (7 $\mu$M) were added to cells, and incubation was performed (15 min, 22°C) at continuous gentle shaking. Cells were washed with and resuspended in the ice-cold AnV-binding medium (25 $\mu$l) and placed in dismountable microchamber for examination under microscope (model BH2, Olympus Europa GmbH, Hamburg, Germany) with 60× water-immersion objective (UPLAPO60 × W, N.A. = 1.2). The U-MSWG epifluorescence filter unit (Olympus Europa GmbH) equipped with 480–550 nm excitation filter, 570 nm dichroic mirror and 590 nm barrier emission filter was used for an observation of AnV and PI fluorescence, whereas the U-MWU filter unit (330–385 nm excitation filter, 400 nm dichroic mirror, 420 nm barrier emission filter) was used for the Hoechst33342 fluorescence observation.

The cell state was recognized on the basis of the specific staining patterns of cells with PI (bright nucleus), AnV (intense uniform membrane staining) and Hoechst33342 (intense staining of condensed chromatin): (− PI, +AnV, +Hoechst33342) and (− PI, − AnV, − Hoechst33342) cells were assigned to apoptotic and living cells, respectively, whereas (+PI, +AnV, +Hoechst33342) and (+PI, − AnV, − Hoechst33342) cells were assigned to the dead cells. The brightness of nonspecific AnV staining of control living cells was taken as a cutoff value to discriminate an apoptosis-related signal. The analysis was performed for 300–500 cells in each experiment and presented as percentage (index) of dead, apoptotic and living cells.

## Measurements of respiratory activity

Activities of cytochrome *c* and its mutants as respiratory-chain electron carriers were monitored by measuring stimulation of respiration (oxygen consumption) of cytochrome *c*-depleted rat liver mitoplasts. Mitoplasts were prepared from rat liver mitochondria as described earlier.[7] Oxygen consumption was measured with a Clark-type $O_2$ electrode. The incubation mixture contained 5 mM Mops/KOH, 0.25 M sucrose, 0.5 mM EGTA, 5 mM succinate, 2 $\mu$M rotenone and mitoplasts (2–3 mg of protein per ml, pH 7.4, 30°C). The respiration rate was measured at the second minute after the addition of cytochrome *c*.

## Confocal spectral imaging measurements

For the CSI measurements, cells were prepared as for the apoptosis assay. In the experiments, where mitochondrial membrane potential was probed, Rh (0.5 $\mu$M) was added to cells together with AnV and PI. For the "visualization" of cytochrome *c* loaded in cells, TR-cytochrome *c* was used for electroporation.

The CSI technique is based on the measurement and analysis of spectra from each point of the selected area of a specimen.[19–21] The CSI measurements were carried out with a device composed of an $Ar^+$ laser (model 164-45, Spectra-Physics GmbH, Darmstadt, Germany), a BH2 microscope, a motorized scanning stage (Marzhauser Wetzlar GmbH, Wetzlar, Germany), a single grating

spectrograph (focal length of 500 mm, 600 grooves/mm plane ruled grating) of OMARS-89 spectrometer (Dilor, Lille, France) and an air-Peltier cooled CCD (Wright Instruments LTD, Enfield, England) with CCD30-11-0-232 sensor (1024 × 256 pixels). Point-by-point scanning of the specimen was carried out with the motorized stage. Excitation and collection of fluorescence signal was performed with the UPLAPO60 × W objective. The collected signal was filtered with a confocal diaphragm and a holographic notch filter (GIPO, Kazan, Russia), dispersed into the spectrum with the grating and detected with CCD. The additional spatial filtration of the signal was performed by reading the spectrum from the selected rows of CCD, which accumulated 70% of the total signal intensity. The lateral, axial and spectral resolutions were $\sim 0.5\ \mu$m, $\sim 3\ \mu$m and 1 nm, respectively. The excitation wavelength was 514.5 nm. The laser power on the sample was $\sim 20\ \mu$W.

The treatment of spectral images was performed as described previously.[19–22] Briefly, each experimental spectrum recorded from the specimen in confocal mode was deconvolved as a sum of reference spectra with weighting coefficients. Taken in the appropriate combination, the fluorescence spectra of AnV, PI, Rh, free TR, TR-cytochrome *c* and the intrinsic cellular fluorescence were used as reference spectra. The integrated intensity of a reference spectrum multiplied by the weighting coefficient for each point produced a two-dimensional map that described the relative distribution of the corresponding dye along the specimen. The mean cellular signals of dyes were calculated for each cell and averaged, when it was required, over 50–90 cells.

The concentration of intracellular TR-cytochrome *c* was deduced from the integrated intensities of intracellular fluorescence spectra of TR-cytochrome *c*. The dependence of fluorescence intensity on TR-cytochrome *c* concentration was calibrated with the solution of TR-cytochrome *c* in PBS under the same experimental conditions (optical pathway, light collection geometry, lateral and axial resolution, laser power and integration time) that were used for the CSI measurements. Quantitative two-dimensional maps of TR-cytochrome *c* distribution were reconstructed for the scanned cells, and the average cytoplasmic concentration of TR-cytochrome *c* was estimated for each individual cell and for 50–90 cell sampling.

## Results

### Apoptosis induced by exogenous cytochrome *c* in WEHI-3 cells

An optimized procedure of electroporation of WEHI-3 cells with cytochrome *c* was developed, which provided an efficient transfer of the protein into the cytosol and a low electroporation-induced damage of cells. Up to 90–97% of cells survived in control experiments after electroporation without cytochrome *c* (Figures 1, upper panel and 2B), whereas the treatment with 10 $\mu$M cytochrome *c* induced an activation of apoptosis in 50–65%

**Figure 1**. Bright-field and epi-fluorescent micrographs of WEHI-3 cells electroporated without cytochrome *c* (upper panel) and with 10 $\mu$M of cytochrome *c* (lower panel) and stained 1 h 45 min after that with AnV, PI and Hoechst33342. Bright staining of nuclei with PI is an indicator of dead cells with permeable membrane (marked with *arrows*). Intense coloration of the plasma membrane with AnV and condensed chromatin with Hoechst33342 are independent indicators of apoptotic cells. Brightness of background fluorescence of living cells settled to black color. In lower panel, non-fluorescent living cells are marked with arrowheads.

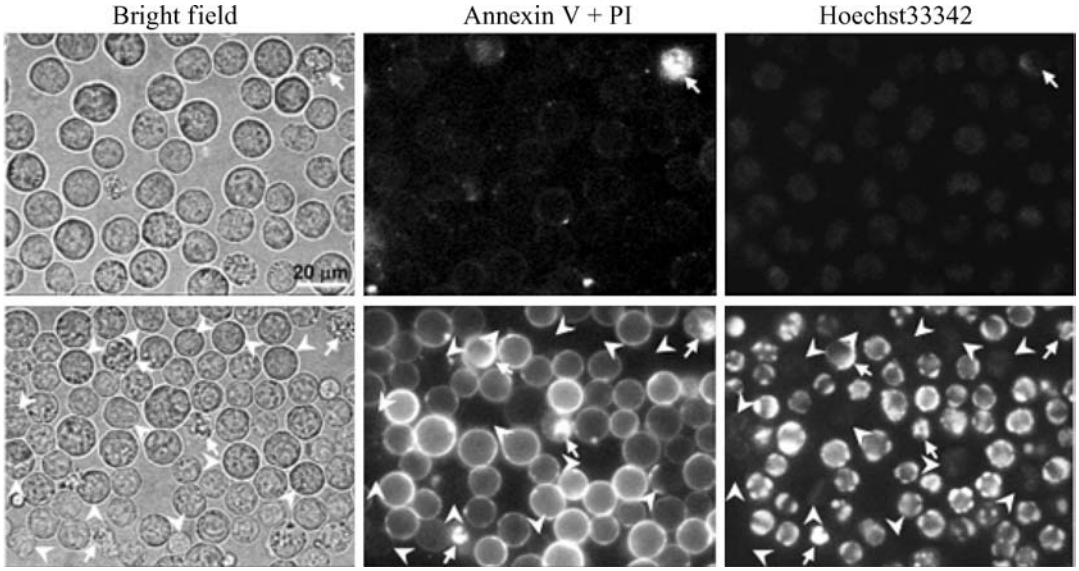
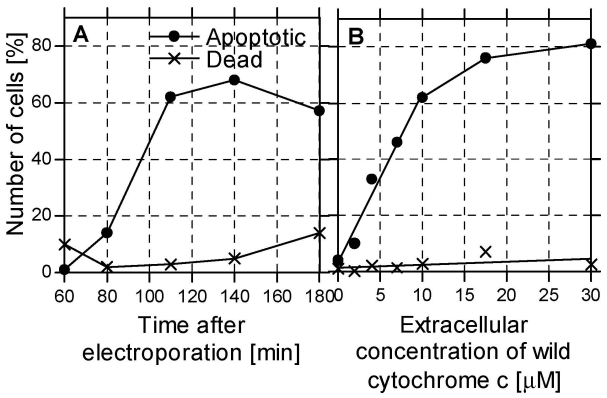


**Figure 2**. Time-course (A) and concentration dependence (B) of apoptosis development. (A) WEHI-3 cells were electroporated with 10 $\mu$M horse heart cytochrome *c* and analyzed with AnV-PI-Hoechst33342 assay at the different time intervals after electroporation. (B) The cells were electroporated with different concentrations of cytochrome *c* and analyzed with AnV-PI- Hoechst33342 assay 1 h 50 min after electroporation.




of cells (Figures 1, lower panel and 2B). Exogenous cytochrome *c* but not the endogenous one was an apoptosis activator, as no apoptosis was detected in cells electroporated without exogenous cytochrome *c*. In these experiments, living, apoptotic and dead cells were easily discriminated with fluorescence microscopy using an AnV-PI- Hoechst33342 assay (Figure 1). Bright staining of nuclei with PI helped to recognize dead cells. Intense coloration of the plasma membrane with AnV and condensed chromatin with Hoechst33342 at the absence of intracellular PI penetration were the signatures of apoptotic cells. Annexin V is a reliable marker of apoptosis[24] because of its selective binding to phoshatidylserine, which is translocated from the inner to the outer leaflet of the plasma membrane in apoptotic cells.[25] The formation of peripheral patches of condensed chromatin is another recognized event of an apoptotic process.

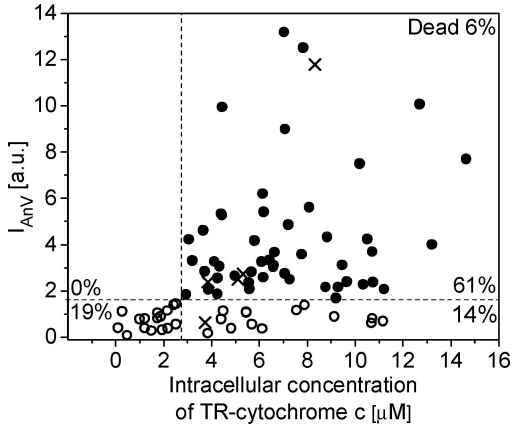
Integrity and functional activity of cytochrome *c* were totally preserved after electroporation: no changes were observed in the absorption spectrum of cytochrome *c* solution after two electric pulses; no electric field-induced breaking of polypeptide chain occurred, since the protein did not pass through protein purifying filter, with a molecular weight limit of 10 kDa. Reutilization of cytochrome *c* solution provided the same level of an apoptotic response of cells.

The time-course of apoptosis development was studied (Figure 2A). A weak or no apoptotic response was detected 1 h after cytochrome *c* injection. An elevated necrotic index was observed because of incomplete cell recovery after electroporation and enhanced susceptibility of cells to mechanical damage during the staining procedure. The number of such sensitized cells was much higher at shorter periods after electroporation (data not shown) and reduced to the background level 1 h 20 min after electroporation. The highest apoptotic index was detected 1h 50 min–2 h 20 min after a cytochrome *c* injection (Figure 2A). An increase in the necrotic index was observed after 3 h (Figure 2A) and most probably indicated the appearance of cells entering the late phase of apoptosis, when the plasma membrane became permeable to PI. For reasons not well understood, the initial rate of apoptosis development varied from one cell flask to the next (from week to week). There were as low as 17% of apoptotic cells 1 h 40 min after a cytochrome *c* injection and 50% just 40 min later at the "slow rate" apoptosis. There were already 40% of apoptotic cells 1 h 40 min after a cytochrome *c* injection and 65% 40 min later at the "high rate" apoptosis. Indeed, the intermediate cases were often observed.

Remarkably, the apoptotic index is linearly dependent on cytochrome *c* concentration in the medium in the 2–10 $\mu$M range and reaches a plateau ($\sim$80% of apoptotic cells) at the 30 $\mu$M concentration of cytochrome *c* (Figure 2B).

Average intracellular concentration of electroinjected cytochrome *c* varies from cell to cell (Figure 3) as measured with the CSI technique for cells loaded with TR-cytochrome *c*. Assuming that penetration of TR-cytochrome *c* in cells is equivalent to that of cytochrome *c*, the cytochrome *c* transfer coefficient, *i.e.* the ratio of the average cytoplasmic concentration of cytochrome *c* to its

**Figure 3**. Representative dot plot of an average intensity of membrane associated AnV fluorescence ($I_{AnV}$) versus average intracellular concentration of electroinjected TR-cytochrome *c*. Average intracellular concentration of TR-cytochrome *c*, $I_{AnV}$ and intensity of nuclear PI fluorescence were measured using the CSI technique 1 h 50 min after electroporation. Cells with $I_{AnV}$ below and above threshold level were classified as living (*open circles*) and apoptotic (*closed circles*), respectively. The threshold level (depicted as a horizontal dashed line) corresponded to 3% of apoptotic cells among the control cells. Cells with detectable nuclear PI fluorescence were considered as dead (*crosses*). Minimal effective TR-cytochrome *c* concentration is presented as a vertical dashed line. Inserted numbers correspond to percentage of non-dead cells in each quadrant restricted by the dashed lines.



concentration in the extracellular medium, was estimated to be $0.3 \pm 0.2$ (mean $\pm$ SD). Indeed, the cytochrome *c* transfer coefficient was independent from the protein concentration (5–20 $\mu$M) in the extracellular medium (data not shown). It should be mentioned that spectral analysis employed in the CSI technique allowed one to discriminate between the overlapping signals of TR-cytochrome *c* and free TR resulted from the conjugate deterioration. Free TR penetrates quickly inside cells even through the intact membrane and has a very high accumulation ratio. It means that a trace amount of free TR in the extracellular medium provides perceptible intracellular concentration of TR. Accordingly correction for contribution of free TR was done in our experiments to avoid overestimation of the intracellular concentration of TR-cytochrome *c*.

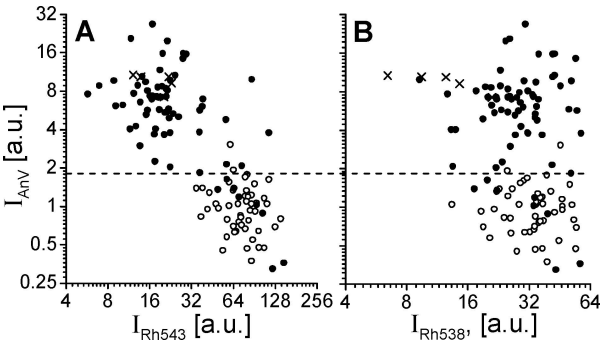
Minimal effective cytoplasmic concentration of cytochrome *c*, which activated apoptosis in WEHI-3 cells, was estimated. In that experiment, the cells were electroinjected with TR-cytochrome *c* and stained with AnV and PI 1 h 50 min after electroporation. Control cells were electroporated without cytochrome *c* and stained with AnV and PI. The CSI technique was used to measure the intracellular concentration of TR-cytochrome *c* and the average intensities of the cell-associated fluorescence of AnV and PI. Overlapped fluorescence signals of free TR, TR-cytochrome *c*, AnV and PI were reliably recognized on the basis of the spectral deconvolution procedure. Intensity of the background fluorescence of AnV bound to control cells was used as a reference level to distinguish between apoptotic and living cells electroporated with cytochrome *c* (Figure 3). The PI signal allowed one to exclude dead cells from the consideration. Finally, the minimal effective cytoplasmic concentration of cytochrome *c* was estimated to be $2.7 \pm 0.5$ $\mu$M. It should be emphasized that many cytochrome *c*-resistant cells were found in which high cytoplasmic concentration of cytochrome *c* was not sufficient for apoptosis activation (Figure 3). Such cells were observed 2, 4 and 6 h after electroporation. This fraction of cytochrome *c*-resistant cells seems to be a reason why 100% apoptosis was not obtained even at the high extracellular concentration of cytochrome *c* (Figure 2B).

To evaluate the state of mitochondria in the course of cytochrome *c*-induced apoptosis, the cells stained with Rh, AnV and PI were studied with the CSI technique 1 h 40 min after cytochrome *c*-electroinjection. Two components of intracellular Rh were discriminated on the basis of differences in the intracellular fluorescence spectra of Rh: (i) the component with a spectrum maximum at 543 nm ($Rh_{543}$), which was mainly present in mitochondria, and (ii) the component with the 538 nm maximum ($Rh_{538}$), which stained the cytoplasm uniformly. The experiments (similar to those described elsewhere[22]) with sodium azide, that induced a decrease in mitochondrial potential, allowed us to recognize the component $Rh_{543}$ as a potential dependent one (data not shown). The intensity of its intracellular signal decreased drastically, when the mitochondrial potential lowered, whereas the intensity of the $Rh_{538}$ signal varied only slightly. Rh is a well-known probe of the mitochondrial potential.[26] We suppose that accumulation of Rh in mitochondria is accompanied with the shift of its fluorescence spectrum to 543 nm, and intensity of $Rh_{543}$ signal is much more sensitive indicator of the mitochondrial potential, than the overall intensity of the Rh intracellular signal.

Using this new approach, an inverse relationship between mitochondrial polarization and phosphatidylserine externalization (an indicator: intensity of AnV signal) in cells with intact membrane (an indicator: no PI signal) was revealed (Figure 4A). It is clearly seen (Figure 4B) that the intensity of the $Rh_{538}$ signal is not responsive to the apoptosis development. The electroporation procedure itself affected neither the overall Rh signal intensity nor the $Rh_{543}$ signal intensity in cells (data not shown). Moreover, cells, in which cytochrome *c* was not able to activate apoptosis (low AnV signal, no PI; Figure 4), maintained a high mitochondrial potential. Therefore, cytochrome *c*-induced apoptosis development is accompanied with a lowering of mitochondrial membrane potential.

From the above experiments it is not clear, whether a loss of potential is due to permeability transition pore opening in mitochondria. There are experimental data[27,28] showing that activated caspases may affect the mitochondria and release mitochondrial apoptotic factors

**Figure 4**. Influence of electroinjected cytochrome *c* on the mitochondrial membrane potential of cells in the course of apoptosis development. Cells were electroporated with (*closed circles*) or without (*open circles*) cytochrome *c* (10 $\mu$M) and stained with AnV, PI and Rh 1 h 40 min after electroporation. Average intracellular fluorescence intensity of two components of Rh, namely, $Rh_{543}$ ($I_{Rh543}$, *A*) and $Rh_{538}$ ($I_{Rh538}$, *B*), average intensity of membrane associated AnV fluorescence ($I_{AnV}$) and intensity of nuclear PI fluorescence were measured using the confocal spectral imaging technique. Cells with $I_{AnV}$ below and above threshold level are considered as living and apoptotic, respectively. The threshold level (depicted as a dashed line) corresponds to 3% of apoptotic cells among the control cells. Cytochrome *c* loaded cells with nuclear PI fluorescence are marked as dead (crosses). Parameters $I_{Rh543}$(*A*) and $I_{Rh538}$(*B*) correspond to mitochondrial potential-sensitive and cytoplasmic component of Rh, respectively.



in the cytosol including endogenous cytochrome *c*, apoptosis inducing-factor, second mitochondria-derived activator of caspase (Smac), mitochondrial protease Omi/HtrA2, endonuclease G and matrix protein Hsp40.[29,30] In our experiments, an incubation of cytochrome *c*-loaded cells (5 or 10 $\mu$M of cytochrome *c*) with cyclosporin A (20 $\mu$M), an inhibitor of permeability transition pore opening,[30] lowered an apoptotic index by factor 1.3–1.5. It indicates, that the apoptotic process initiated with cytochrome *c* induces permeability transition pore opening, and the mitochondrial factors enhance and/or accelerate the apoptosis development.

## Apoptotic activity of cytochrome *c* mutants

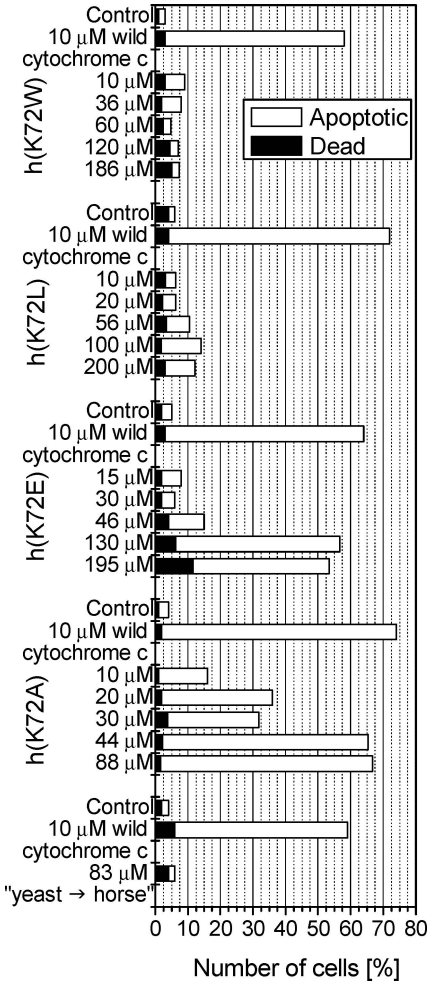
Apoptotic activity of four K72-substituted mutants of cytochrome *c* and "yeast → horse" hybrid cytochrome *c* was studied in living WEHI-3 cells (Figure 5) using the electroporation technique and AnV-PI-Hoechst33342 assay described above. The h(K72A) activity was moderately reduced as compared to that of wild cytochrome *c*. The K72E substitution decreased noticeably an apoptogenic effect, but apoptosis was still observed at the high h(K72E) concentration. The result of the K72L substitution was still stronger: even the high h(K72L) concentration induced only a weak apoptotic response of cells. No apoptogenic effect was observed with h(K72W) and "yeast → horse" hybrid cytochrome *c*.

To describe quantitatively the differences in the activities between wild cytochrome *c*, h(K72A), h(K72E) and h(K72L), we have calculated the equieffective concentrations of wild cytochrome *c*, *i.e.* the concentrations of wild cytochrome *c* required to produce the same apoptotic index as definite concentrations of the mutants produced. The decrease in apoptotic activity (*i.e.* the ratio of the active concentration of mutant to the equieffective concentration of wild cytochrome *c*) was ~ fivefold for h(K72A), ~70-fold for h(K72L) and ~15-fold for h(K72E). Even if existed, apoptotic activity of "yeast → horse" hybrid cytochrome *c* and h(K72W) was at least 200-fold lower than that of wild horse heart cytochrome *c*.

## h(K72W) inhibits the proapoptotic activity of wild cytochrome *c*

Ability of h(K72W) to inhibit activity of wild cytochrome *c* was tested by loading the cells with a mixture of h(K72W) and wild cytochrome *c* at different h(K72W)/cytochrome *c* molar ratios but constant concentration of wild cytochrome *c*. A decrease in apoptotic index was observed at the increase in the h(K72W)/cytochrome *c* molar ratio (Figure 6). One may speculate that h(K72W) binds with Apaf-1 competitively to wild cytochrome *c*.

**Figure 5**. Apoptotic activities of wild cytochrome *c*, h(K72W), h(K72L), h(K72E), h(K72A) and "yeast → horse" hybrid electroinjected in living WEHI-3 cells. Cells were electroporated without cytochrome *c* (*control*), with 10 $\mu$M of wild cytochrome *c* as a positive control or with denoted concentration of corresponding mutant variant. Measurements were performed 110 min after electroporation. Black and white bars represent the percentages of dead (+PI) and apoptotic (−PI, +AnV, +Hoechst33342) cells, respectively.




Apparently, the Apaf-1 binding ability of h(K72W) is lower than that of wild cytochrome *c*, since high excess of h(K72W) is required to inhibit the wild cytochrome *c* apoptotic activity (Figure 6).

## Respiratory activities of cytochrome *c* mutants

Preservation of the electron transfer activity by K72A-, K72G-, K72R- and K72L- substituted cytochrome *c* mutants was demonstrated earlier.[7] Similarly, the K72E- and K72W-substitutions did not affect the electron transfer efficacy of the horse heart cytochrome *c* (Figure 7). Under the same conditions the efficacy of yeast cytochrome *c* was

**Figure 6**. An inhibition of wild cytochrome *c*-induced apoptosis by h(K72W). Cells were electroporated without cytochrome *c* (*control*), with 5 $\mu$M of wild cytochrome *c* or with a mixture of wild cytochrome *c* and h(K72W) mutant at different molar ratios. Black and white bars represent a percentage of dead (+PI) and apoptotic (−PI, +AnV, +Hoechst33342) cells, respectively.

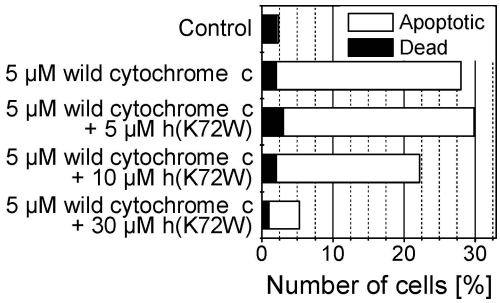
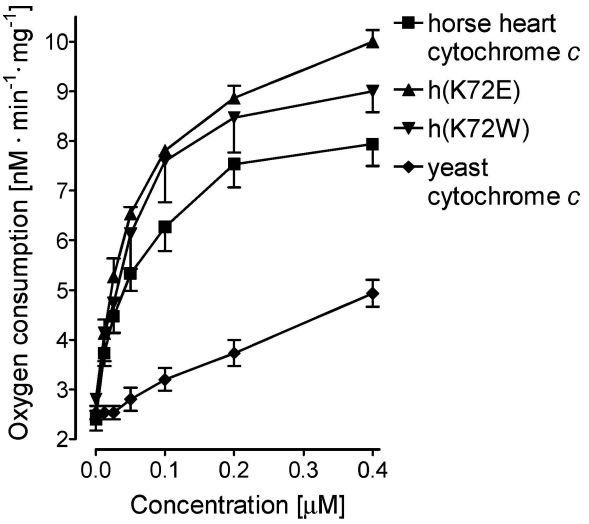



**Figure 7**. Stimulation of respiration of cytochrome *c*-deficient mitoplasts by various cytochrome *c* species. Stimulation of respiration was estimated on the basis of an increase in the rate of oxygen consumption per mg of mitochondrial protein at the different concentrations of added horse heart cytochrome *c*, yeast cytochrome *c*, h(K72E) and h(K72W).




significantly lower (Figure 7), whereas "yeast → horse" hybrid approached noticeably to horse heart cytochrome *c* in this parameter.[7]

## Discussion

The *in situ* apoptosis system described in this paper has a major advantage: it allows one to deliver efficiently exogenous pro-apoptotic proteins to the cytoplasm of living cells and activate apoptosis without concomitant damage of cells induced by the loading procedure itself (Figures 1 and 2). Like some other investigators,[9–12] we have used an electroporation technique to introduce cytochrome *c* into cells, but due to a careful optimization of the procedure, we have eliminated cell trauma. Under our conditions, up to 80% of WEHI-3 cells demonstrated an apoptotic response to cytochrome *c* electroinjection, and a very low percentage of cells were positively (dead or apoptotic) responsive to the blank electroinjection (Figure 2). For comparison, 40–50% and 25–30% of Jurkat cells gave the response to electroporation with and without cytochrome *c*, respectively, in the assay described previously.[12] As reported elsewhere,[11] up to 20% of Jurkat cells entered in apoptosis and 20–30% of cells were dead after electroporation with cytochrome *c* while the electroporation procedure itself resulted in 3% of apoptotic and 21% of dead cells. In the pioneering work on electroporation of cells with cytochrome *c*,[9] 20 to 50% of the cells lysed after the procedure, and ~25–38% of the survived cells were recognized as apoptotic after electroinjection of cytochrome *c*. The electroporation conditions were optimized later on to reduce cell death down to 11–20% and to achieve bovine serum albumin loading in 45–80% of leukemic cells of different origin.[10] The electroporation procedures used elsewhere[9–12] were good enough to get a definite "yes/no" answer, whereas our assay permits quantitative experiments.

The relatively fast (1.5 h) development of cytochrome *c* induced apoptosis observed with our assay is consistent with the results of earlier studies[13] in which (i) caspase activation was detected in lysates prepared from U937 and Jurkat cells 10 min after pinocytic loading of cytochrome *c* and (ii) caspase activity peaked at 1–2 h following loading. Our data indicate that development of apoptosis in WEHI-3 cells achieves the stage of phosphatidylserine externalization and chromatin aggregation during ~1.5 h after cytochrome *c* injection (Figures 1 and 2).

Linear dependence of the apoptotic cell percentage on the extracellular cytochrome *c* concentration (2–10 $\mu$M, Figure 2B) can be explained as follows. A cell goes to apoptosis when the cytoplasmic concentration of cytochrome *c* overcomes a "competence-to-die" value. Intracellular penetration of exogenous cytochrome *c* occurs due to diffusion through the transient pores formed by the electric pulse in the plasma membrane. The resultant cytoplasmic concentration depends on the number of pores per cell, their diameter and lifetime. Dispersion of these parameters from cell to cell is a main reason for the observed distribution profile of the intracellular concentration of electroinjected cytochrome *c* (Figure 3). According to diffusion theory, intracellular concentration has to be linearly dependent on the extracellular concentration of cytochrome *c*, if all the parameters of pores are constant. As a result, an increase in the extracellular concentration of cytochrome *c* shifts the distribution profile as a whole to the higher values, thus increasing the number of cells, in which the cytoplasmic concentration of cytochrome *c* is high enough to activate apoptosis. As shown above, the cytochrome *c*

transfer coefficient was independent from the protein concentration (5–20 $\mu$M) in the extracellular medium thus confirming the validity of such consideration.

It should be emphasized that cytochrome *c*-resistant WEHI-3 cells were found, in which a high cytoplasmic concentration of cytochrome *c* was not sufficient for apoptosis activation (Figure 3). This may depend on the balance of intracellular pro- and anti-apoptotic factors in different cells. Among antiapoptotic factors, the inhibitor of apoptosis proteins (IAP) such as hILP, XIAP, cIAP-1, survivin, and Bruce can be mentioned. Human IAP-like protein (hILP) was reported to protect cells from apoptosis induced by the microinjection of cytochrome *c*.[18] Neutrophils have a lowered threshold requirement for cytochrome *c* in the Apaf-1-dependent cell death pathway.[31] Highly elevated Apaf-1 levels and abnormally low levels of IAP proteins were concluded to promote apoptosis activation with very low concentration of cytochrome *c* in the neutrophils.

Previously, a certain proportion of cells, which displayed no apoptotic response, was supposed to result from failed electroinjection[9] or pinocytic loading[13] of cytochrome *c* in some cells. We speculate that a fraction of cytochrome *c*-resistant cells is present in different cell lines and at least partially accounts for the restricted response of cells to cytochrome *c* in the cited works. Data on sensitivity of various cell lines to the cytochrome *c* induced apoptosis are summarized in Table 1. So far, more than 99% apoptotic response was reported for the clone 8 embryonal mouse fibroblasts only,[17] whereas some cell types were found to be of relatively low sensitivity, or even resistant, to the cytochrome *c* pro-apoptotic stimulus (Table 1). Besides the question about the origin of differences in the apoptotic response, this set of data indicates that the cells should be carefully selected, when developing a model *in situ* system for the apoptosis investigation. We know from experience that WEHI-3 cells surpass the Jurkat, HL60 and K562 cells in the apoptotic response.

There are controversial data concerning the influence of exogenous cytochrome *c* delivered to cytoplasm on mitochondrial transmembrane potential: no effect of electroinjected cytochrome *c*;[9] no effect of microinjected cytochrome *c* until immediately before cell death, when mitochondria loose membrane potential;[16] specifically induced mitochondrial depolarization owing to pinocytically loaded[13] or electroinjected[11] cytochrome *c*. Our data confirm the response of mitochondria to exogenous cytochrome *c*: development of apoptosis initiated with electroinjected cytochrome *c* is accompanied with loss of mitochondrial potential (Figure 4A).

It should be stressed that the observed decrease in potential is not a direct effect of exogenous cytochrome *c* on mitochondria since a fraction of non-apoptotic cells having high cytoplasmic concentration of cytochrome *c* was detected (Figure 3). Caspases or some cytosolic component(s)[32] activated by cytochrome *c* seem to be responsible for a lowering of the mitochondrial membrane potential.

**Table 1**. Apoptotic responsivity of different cells loaded with exogenous cytochrome *c*

| Cell type | Responsivity to exogenous cytochrome *c* |
|---|---|
| Human T-cell lymphoblastoid Jurkat | yes[11–13] |
| Human myelo-monocytic U-937 | yes[13] |
| Human lymphoblastoid SKW6 | yes[12] |
| Human embryonic kidney 293 | yes[8] |
| Human cervical carcinoma HeLa | weak[8] |
| Murine interleukin-3 dependent pro-B (Bo) | yes[9] |
| Murine interleukin-3 dependent A15 | yes[9] |
| Murine interleukin-3 dependent B15 | yes[9] |
| Murine myelo-monocytic WEHI-3b | yes[9] |
| Murine adrenocortical tumor cells Y1 | yes[17] |
| Murine Swiss 3T3 fibroblasts | yes[17] |
| Rat promyelocytic leukemia IPC-81 | weak[17] |
| Human lymphoma CEM | no[9] |
| Breast carcinoma MCF-7 | no[8] |
| Clone 8 embryonal mouse fibroblasts | yes[17] |
| Normal rat kidney NRK | yes[17] |
| Peripheral blood mononuclear cells from healthy donors | yes[10] |
| Chronic lymphocytic leukemia cells from patients | yes[10] |

We have tried to estimate an apoptosis-inducing "power" of cytochrome *c*, as a basis for further comparison of the pro-apoptotic ability of different mitochondrial factors. The minimum effective intracellular concentration of cytochrome *c* in WEHI-3 cells is equal to $2.7 \pm 0.5$ $\mu$M that corresponds to $47 \pm 9$ fg of cytochrome *c* per cell. Our estimation is based on the measurement of cytochrome *c* concentration delivered to a living cell (Figure 3).

It was reported that pinocytic loading of $\sim 12$ and 16 fg of cytochrome *c* per cell exert specific pro-apoptotic effect in Jurkat and U937 cells, respectively.[13] Keeping in mind that the analyzed lysates were prepared from different (living, apoptotic and, probably, dead) cells, these values can be considered as a very approximate low estimation of the apoptosis-activating amount of cytochrome *c* in the above cell types. Microinjection of kidney 293 cells with 120 fg/cell cytochrome *c* corresponded to a threshold of apoptosis activation,[8] but, in fact, the delivered amount of cytochrome *c* was not exactly known. It was estimated to range from 12 to 120 fg/cell.

Assuming that the mitochondrial content of cytochrome *c* in WEHI-3 cells is comparable to that in

HeLa cells (10 $\mu$M if uniformly distributed throughout cytoplasm[33]), kidney 293 and MCF 7 cells (40–100 fg/cell[8]), we conclude that mitochondrial capacity of cytochrome *c* is approximately equal to the value required for apoptosis activation. It means that (i) moderate variations in concentration of the apoptosis-inhibiting factors may considerably affect the cytochrome *c* related apoptotic effect; (ii) synergetic action of some pro-apoptotic factors is required to overcome cellular "competence-to-die" barrier.

"Yeast $\rightarrow$ horse" hybrid created by introducing S2D, K4E, A7K, T8K, and K11V substitutions and deleting five N-terminal aminoacid residues in yeast cytochrome *c* possesses no pro-apoptotic activity in the living cells (Figure 5). This fact is consistent with the data obtained earlier[7] using Xenopus egg and mammalian cell extracts, where this hybrid was inactive in inducing apoptosis.

Our quantitative analysis of pro-apoptotic activity of cytochrome *c* mutants in living cells confirms that single mutations at the position 72 diminish the apoptogenic effect of cytochrome *c*. In Xenopus egg extracts, the decrease in caspase activation was twofold for h(K72R), three- to fourfold for h(K72G) and 12-fold for h(K72A) and h(K72L).[7] Similar data were obtained in mammalian cell extracts.[7] It is not clear, why no caspase-9 activation was detected with the h(K72A) variant in the *in vitro* reconstitution system.[5] Our data indicate that h(K72A) possesses considerable proapoptogenic activity in a cellular environment, just fivefold lower than that of wild cytochrome *c*. The decrease in apoptotic activity of h(K72L) is even higher in a cellular environment (70-fold), than *in vitro* (12-fold). The K72E substitution is fivefold less harmful for apoptotic function of cytochrome *c* in cells, than the K72L substitution. The apoptotic activity of cytochrome *c* is in fact abolished by the K72W substitution (Figure 5). Besides the mutated cytochrome *c* having a K72 residue with a trimethylated e-amino group,[6] h(K72W) is the second variant of mammalian cytochrome *c*, where site-directed modification of single residue blocks totally the apoptotic function of cytochrome *c*.

Our results suggest that h(K72W) competes with wild cytochrome *c* for activation of apoptosis in the living cells (Figure 6). Affinity of h(K72W) for Apaf-1 seems to be severalfold lower than that of wild cytochrome *c*, since 50% inhibitory concentration of h(K72W) ($\sim$15 $\mu$M) is threefold higher than the applied (5 $\mu$M) concentration of wild cytochrome *c* (Figure 6). One can expect that the apoptotic activity of h(K72W) should be also just severalfold lower than that of wild cytochrome *c*, but it is at least 200-fold lower. Therefore the complexes of Apaf-1 with h(K72W) are defective with respect to interaction with procaspase-9 and/or dATP. This seems to be an additional important factor contributing to the overall negligible apoptotic activity of h(K72W).

Our results are in contrast with the data on inability of cytochrome *c* with trimethylated K72 residue to compete with wild cytochrome *c* for the binding with Apaf-1 in cell extracts.[6] It was also reported that the caspase-9 activating ability of K72-trimethylated cytochrome *c* was very low.[5] Thus, trimethylation of the K72 residue seems to be more harmful to Apaf-1 binding than K72W substitution.

Apparently, high affinity ($K \sim 4 \times 10^7\ M^{-1}$) of Apaf-1 for cytochrome *c* demonstrated using Zn-substituted cytochrome *c*[34] cannot be provided with K72 residue solely, even if this residue is essential for the apoptotic function of cytochrome *c*. Cytochrome *c*-Apaf-1 recognition involves a large region of cytochrome *c* surface, and many residues are important for this interaction.[5] The interacting residues are situated at the opposite sides of cytochrome *c* globule indicating that the protein is almost fully "wrapped" by Apaf-1.[5] It seems that cytochrome *c* binding induces dATP/ATP assisted conformational changes in Apaf-1[35] due to concerted electrostatic interactions of the surface charged residues. The contribution of individual charged residues is almost additive in many cases.[5] Previously published[5,7] and our data indicate that a change in the charge sign of residue 72 (K72E) or introduction of hydrophobic side chain (K72A, K72L, K72W) affect considerably proapoptotic activity of cytochrome *c*. Long and/or bulk hydrophobic side chain (Leu, Trp) is more disturbing than short one (Ala). Exact conformation of the positively charged side chain (K72R)[7] or the charge itself (K72G)[7] has a weak influence on the formation and functional activity of cytochrome *c*-Apaf-1 complex.

In this context, it is noteworthy that yeast iso-1-cytochrome *c* and some of its mutants, which have many differences in aminoacid sequence as compared with horse heart cytochrome *c*, possess low Apaf-1 binding ability in cell extracts.[6] Very low affinity of yeast cytochrome *c* for Apaf-1 was demonstrated with fluorescence polarization spectroscopy.[5] On the other hand, a categorical conclusion concerning low affinity for Apaf-1 of various mutants of horse and yeast cytochrome *c*,[5] which is based on low caspase-activating ability, is a rather simplified consideration of the problem, since the mismatched participation of mutated residues in functional activity of the cytochrome *c*-Apaf-1 complex is ignored.

The "yeast $\rightarrow$ horse" hybrid and all the studied K72-substituted mutants of horse heart cytochrome,[7] including h(K72W) and h(K72E) characterized in this work, support respiration. Thus, h(K72W) lost pro-apoptotic activity, but it is still able to function in the mitochondrial respiratory chain. In this connection, the creation of cells transfected to express K72W-substituted cytochrome *c* may offer a new instrument for investigation of apoptosis in the living system with inactivated apoptotic function of cytochrome *c*.

## Conclusion

Duly optimized electroporation procedure is a promising, highly efficient and suitable tool for the transfer of exogenous proteins in the cytoplasm in order to investigate their function in living cells. In particular, this approach provides a quantitative comparison of apoptotic activity of cytochrome *c* mutants and helps to clarify their intracellular molecular interactions. Experiments with living cells confirm that single residue mutations at position 72 decrease the pro-apoptotic function of cytochrome *c*. The K72W-substituted cytochrome *c* is competent as a respiratory-chain electron carrier but possesses reduced affinity for Apaf-1 and forms an inactive complex. This mutant is well suited for knock-in studies of the role of cytochrome *c* -mediated apoptosis.

### Acknowledgments

This research was supported by grants 43.106.11.0018 from Ministry of Science and Technology of Russian Federation, 01.106.11.0006 from RF Federal Agency for Science and Innovations and the RAS program on molecular and cellular biology.

### References

1. Liu X, Naekyung C, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: Requirement for dATP and cytochrome *c*. *Cell* 1996; **86**: 147–157.
2. Kluck RM, Martin SJ, Hoffman BM, Zhou JS, Green DR, Newmeyer DD. The release of cytochrome *c* from mitochondria: A primary site for Bcl-2 regulation of apoptosis. *EMBO J* 1997; **16**: 4639–4649.
3. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X. Apaf-1, a human protein homologous to *C* elegans CED-4, participates in cytochrome *c*-dependent activation of caspase-3. *Cell* 1997; **90**: 405–413.
4. Li P, Nijhawan D, Budihardjo I, *et al.* Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; **91**: 479–489.
5. Yu T, Wang X, Purring-Koch C, Wei Y, McLendon GL. A mutation epitope for cytochrome *c* binding to the apoptosis protease activation factor-1. *J Biol Chem* 2001; **276**: 13034–13038.
6. Kluck RM, Ellerby LM, Ellerby HM, *et al.* Determinants of cytochrome *c* pro-apoptotic activity The role of lysine 72 trimethylation. *J Biol Chem* 2000; **275**: 16127–16133.
7. Abdullaev ZKh, Bodrova ME, Chernyak BV, *et al.* A cytochrome *c* mutant with high electron transfer and antioxidant activities but devoid of apoptogenic effect. *Biochem J* 2002; **362**: 749–754.
8. Li F, Srinivasan A, Wang Y, Armstrong RC, Tomaselli KJ, Fritz LC. Cell-specific induction of apoptosis by microinjection of cytochrome *c* Bcl-xL has activity independent of cytochrome *c* release. *J Biol Chem* 1997; **272**: 30299–30305.
9. Garland JM, Rudin C. Cytochrome *c* induces caspase-dependent apoptosis in intact hematopoietic cells and overrides apoptosis suppression mediated by bcl-2, growth factor signaling, MAP-kinase-kinase, and malignant change. *Blood* 1998; **92**: 1235–1246.
10. Eksioglu-Demiralp E, Kitada S, Carson D, Garland J, Andreef M, Reed JC. A method for functional evaluation of caspase activation pathways in intact lymphoid cells using electroporation mediated protein delivery and flow cytometric analysis. *J Immun Meth* 2003; **275**: 41–56.
11. Gabriel B, Sureau F, Casselyn M, Teissie J, Petit PX. Retroactive pathway involving mitochondria in electroloaded cytochrome *c* -induced apoptosis. Protective properties of Bcl-2 and Bcl-XL. *Exp Cell Res* 2003; **289**:195–210.
12. Vier J, Linsinger G, Hacker G. Cytochrome *c* is dispensable for fas-induced caspase activation and apoptosis. *Biochem Biophys Res Commun* 1999; **261**: 71–78.
13. Gilmore KJ, Quinn HE, Wilson MR. Pinocytic loading of cytochrome *c* into intact cells specifically induces caspase-dependent permeabilization of mitochondria: Evidence for a cytochrome *c* feedback loop. *Cell Death Differ* 2001; **8**: 631–639.
14. Deshmukh M, Johnson EM. Evidence of a novel event during neuronal death—development of competence-to-die in response to cytoplasmic cytochrome *c*. *Neuron* 1998; **21**: 695–705.
15. Bhuyan AK, Varshney A, Mathew MK. Resting membrane potential as a marker of apoptosis: Studies on Xenopus oocytes microinjected with cytochrome *c*. *Cell Death Differ* 2001; **8**: 63–69.
16. Chang S, Phelps P, Berezesky I, Ebersberger M, Trump B. Studies on the mechanisms and kinetics of apoptosis induced by microinjection of cytochrome *c* in rat kidney tubule epithelial cells (NRK-52E). *Am J Path* 2000; **156**: 637–649.
17. Brustugun OT, Fladmark KE, Doskeland SO, Orrenius S, Zhivotovsky B. Apoptosis induced by microinjection of cytochrome *c* is caspase-dependent and is inhibited by Bcl-2. *Cell Death Differ* 1998; **5**: 660–668.
18. Duckett CS, Li F, Wang Y, Tomaselli KJ, Thompson CB, Armstrong RC. Human IAP-like protein regulates programmed cell death downstream of Bcl-$x_L$ and cytochrome *c*. *Mol Cell Biol* 1998; **18**: 608–615.
19. Feofanov A, Charonov S, Kudelina I, Fleury F, Nabiev I. Localization and molecular interactions of mitoxantrone within living K562 cells as probed by confocal spectral imaging analysis. *Biophys J* 1997; **73**: 3317–3327.
20. Feofanov A, Charonov S, Fleury F, Kudelina I, Nabiev I. Quantitative confocal spectral imaging analysis of mitoxantrone within living K562 cells: Intracellular accumulation and distribution of monomers, aggregates, naphtoquinoxaline metabolite and drug-target complexes. *Biophys J* 1997; **73**: 3328–3336.
21. Feofanov AV, Grichine AI, Kudelina IA, *et al.* Study of the localization and molecular interactions of biologically active compounds in living cells and tissue slices by the confocal spectral imaging technique. *Russian J Bioorganic Chem* 1999; **25**: 793–802.
22. Feofanov A, Sharonov G , Grichine A, *et al.* Comparative study of photodynamic properties of 13,15-N-cycloimide derivatives of chlorin p6. *Photochem Photobiol* 2004; **79**: 172–188.
23. Dolgikh DA, Latypov PF, Abdullaev ZK, Kolov V, Roder H, Kirpichnikov MP. Expression of mutant horse cytochrome *c* genes in Escherichia coli. *Russ J Bioorg Chem* 1998; **24**: 672–675.
24. Bedner E, Li X, Gorczyca W, Melamed MR, Darzynkiewicz Z. Analysis of apoptosis by laser scanning cytometry. *Cytometry* 1999; **35**: 181–195.

25. Martin SJ, Reutelingsperger CP, McGahon AJ, *et al.* Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: Inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 1995; **182**:1545–1556.
26. Chen LB. Fluorescent Labeling of Mitochondria. In: Wang Y-L, Lansing TD, eds. *Fluorescent Microscopy of Living Cells in Culture*. Part A. San Diego: Academic Press, Inc. 1989: 103–123.
27. Marzo I, Susin SA, Petit PX, *et al.* Caspases disrupt mitochondrial membrane barrier function. *FEBS Lett* 1998; **427**:198–202.
28. Cosulich SC, Savory PJ, Clarke PR. Bcl-2 regulates amplification of caspase activation by cytochrome *c*. *Curr Biol* 1999; **9**: 147–150.
29. Saelens X, Festjens N, Walle LV, van Gurp M, van Loo G, Vanderbale P. Toxic proteins released from mitochondria in cell death. *Oncogene* 2004; **23**: 2861–2874.
30. Chandra D, Tang DG. Mitochondrially localized active caspase-9 and caspase-3 result mostly from translocation from the cytosol and partly from caspase-mediated activation in the organelle. *J Biol Chem* 2003; **278**:17408–17420.
31. Murphy BM, O'Neill AJ, Adrain C, Watson RWG, Martin SJ. The apoptosome pathway to caspase activation in primary human neutrophils exhibits dramatically reduced requirements for cytochrome *c*. *J Exp Med* 2003; **197**: 625–632.
32. Bossy-Wetzel E, Green DR. Caspases induce cytochrome *c* release from mitochondria by activating cytosolic factors. *J Biol Chem* 1999; **274**: 17484–17490.
33. Waterhouse NJ, Goldstein JC, von Ahsen O, Schuler M, Newmeyer DD, Green DR. Cytochrome *c* maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process. *J Biol Chem* 2001; **153**: 319–328.
34. Purring-Koch C, McLendon G. Cytochrome *c* binding to Apaf-1: The effects of dATP and ionic strength. *Proc Natl Acad Sci USA* 2000; **97**: 11928–11931.
35. Hu Y, Benedict MA, Ding L, Nunez G. Role of cytochrome *c* and dATP/ATP hydrolysis in Apaf-1-mediated caspase-9 activation and apoptosis. *EMBO J* 1999; **18**: 3586–3595.