Cytochrome c is released from mitochondria into the cytosol in cells undergoing apoptosis. The temporal relationship between cytochrome c release and loss of mitochondrial membrane potential was monitored by laser-scanning confocal microscopy in single living pheochromocytoma-6 cells undergoing apoptosis induced by staurosporine. Mitochondrial membrane potential monitored by tetramethylrhodamine methyl ester decreased abruptly in individual cells from 2 to 7 h after treatment with staurosporine. Depolarization was accompanied by cytochrome c release documented by release of transfected green fluorescent protein-tagged cytochrome c in these cells. The results show that mitochondrial depolarization accompanies cytochrome c release in pheochromocytoma-6 cells undergoing apoptosis.

Substantial evidence implicates mitochondria in apoptotic cell death (1–4). It is thought that proteins normally restricted to the mitochondrial intermembrane space, including cytochrome c and the 50-kDa apoptosis-inducing factor, are released to the cytosol where they initiate the apoptotic cascade (5–8). The mechanism of protein release has not been established. One proposal is that protein release requires rupture of the outer mitochondrial membrane and that this is a consequence of the onset of the mitochondrial permeability transition (MPT) (9, 10). Onset of the MPT, which is an inner membrane process, would depolarize the inner membrane (11). Thus, the MPT hypothesis implies a direct temporal relationship between mitochondrial depolarization and cytochrome c release. Experimental data addressing this connection are limited.

 Actually, two recent studies showed that cytochrome c release during apoptosis was not accompanied by mitochondrial depolarization (12, 13). In these studies, mitochondrial membrane potential (Δψ) was monitored by rhodamine 123 and DiOC6, compounds that are known to inhibit the ATP synthase before (21, 22). PC6 cells were harvested, washed twice with ice-cold phosphate-buffered saline, and lysed with ice-cold lysis buffer (150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 50 mM Tris-HCl, pH 8.0) containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride). The cell lysates were centrifuged at 10,000 g for 10 min at 4 °C. Supernatants were mixed with Laemmli sample buffer. Forty μg of protein each of nontransfected cells and cytochrome c-GFP transfected cells, and 1 μg of protein of GFP were separated on a 12% SDS-polyacrylamide gel electrophoresis gel. The proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) and blotted with primary monoclonal anti-GFP antibody (CLONTECH Laboratories, Inc., Palo Alto, CA). Peroxidase activity was developed with ECL (Amersham Pharmacia Biotech).

**Confocal Microscopy**—To investigate localization of GFP and GFP-fusion proteins, cells were transferred to a mounting chamber on the microscope stage in Dulbecco’s modified Eagle’s medium/F12 medium supplemented with 15 mM HEPES and 5% fetal bovine serum at pH 7.2.

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after 66 h of transfection. Subsequently, cells were loaded with 40 nM MitoTracker Red CMXRs. Green fluorescence of cytochrome c-GFP (A and A') and GFP (B and B') was visualized by confocal microscopy. Cytochrome c-GFP displayed a punctate pattern of fluorescence (A) that matches that of MitoTracker Red CMXRs (A'), whereas GFP fluorescence (B) was diffuse and did not match MitoTracker Red (B'). Scale bar, 10 μm. C, Western blot of cytochrome c-GFP and GFP. In cells transfected with the cytochrome c-GFP construct, expression of the expected 43-kDa fusion protein band was revealed. By contrast, transfection of native GFP cDNA led to expression of a 30-kDa protein, which is close to the molecular weight of GFP.

**RESULTS AND DISCUSSION**

Rat PC6 cells were transiently transfected with plasmids bearing the cytochrome c-GFP fusion construct. To confirm that cytochrome c-GFP was targeted to mitochondria, cells were co-loaded with Mitotracker Red CMXRs, which localizes to mitochondria (23). Cytochrome c-GFP and Mitotracker Red CMXRs were imaged using laser scanning confocal microscopy. As shown in Fig. 1A, cytochrome c-GFP displayed a punctate pattern of fluorescence that was indistinguishable from that of Mitotracker Red. These confocal images demonstrated that the cytochrome c-GFP fusion protein was localized to mitochondria (Fig. 1, A and A'). By contrast, cells transfected with GFP cDNA alone displayed a diffuse fluorescence (Fig. 1B). The expression of the cytochrome c-GFP fusion protein was also confirmed by Western blot analysis using anti-GFP antibody (21, 22) (Fig. 1C).

To monitor changes in mitochondrial Δψ, cytochrome c-GFP-
transfected cells were loaded with TMRM. TMRM is a cationic fluorophore that accumulates electrophoretically into mitochondria in response to the negative mitochondrial Δψ (20). When mitochondria depolarize, they release TMRM and the red fluorescence inside mitochondria disappears. Accordingly, the green fluorescence of cytochrome c-GFP and the red fluorescence of TMRM were monitored simultaneously in single living cells by laser scanning confocal microscopy. Fig. 2A shows a field of PC6 cells transfected with cytochrome c-GFP (right panel) and loaded with TMRM (left panel). Before addition of staurosporine to induce apoptosis, all cells in the field displayed high mitochondrial Δψ, as indicated by bright punctate fluorescence of TMRM (Fig. 2A, 0 h, left panel). Not all cells were transfected with cytochrome c-GFP, as indicated by the absence of green fluorescence, but all viable cells had polarized mitochondria as revealed by red fluorescence (Fig. 2A, 0 h, left panel).

To induce apoptosis, cells were treated with 5 μM staurospo-
rime, a protein kinase inhibitor, which induces apoptosis in a wide variety of cell types (12, 17, 25, 26). In the field of PC6

to confirm that the changes we observed regarding mitochon-
drial depolarization and cytochrome c-GFP release were indeed related to staurosporine treatment, we observed

to cytochrome c release.

To quantitate the changes in TMRM fluorescence over time, we measured total TMRM fluorescence in each cell. Fig. 3A

Our fusion construct is a 43-kDa protein, whereas endoge-
nous cytochrome c is a 15-kDa protein. Thus, endogenous cy-
tochrome c might be released before mitochondrial depolariza-
tion and before release of the larger molecular weight fusion

Our results show directly for the first time that mitochondrial depolarization and cytochrome c release occur simulta-

When cytochrome c-GFP was released from the mitochondria into the cytosol, green fluorescence increased in the nucleus in all cells studied. In PC6 cells, the cytosol is a small proportion of cell volume that is mostly comprised of nucleoplasm. Once released from mitochondria into the cytosol, cytochrome c-GFP rapidly diffused into the nucleus, presumably through the nuclear pores. Thus, increases of nuclear cytochrome c-GFP reflected increases of cytosolic cytochrome c-GFP. The impor-
tance of this nuclear cytochrome c-GFP in the apoptotic process remains to be elucidated.

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