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ATP-dependent Steps in Apoptotic Signal Transduction[1]

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ABSTRACT

Apoptotic changes of the nucleus induced by Fas (Apo1/CD95) stimulation are completely blocked by reducing intracellular ATP level. In this study, we examined the ATP-dependent step(s) of Fas-mediated apoptotic signal transduction using two cell lines. In SKW6.4 (type I) cells characterized by rapid formation of the death-inducing signaling complex on Fas treatment, the activation of caspases 8, 9, and 3, cleavage of DFF45 (ICAD), and release of cytochrome c from the mitochondria to the cytoplasm were not affected by reduction of intracellular ATP, although chromatin condensation and nuclear fragmentation were inhibited. On the other hand, in the Fas-mediated apoptosis of Jurkat (type II) cells, which is characterized by involvement of mitochondria and, thus, shares signal transduction mechanisms with apoptosis induced by other stimuli such as genotoxins, activation of the three caspases, cleavage of DFF45 (ICAD), and nuclear changes were blocked by reduction of intracellular ATP, whereas release of cytochrome *c* was not affected. These results suggested that the ATP-dependent step(s) of Fas-mediated apoptotic signal transduction in type I cells are only located downstream of caspase 3 activation, whereas the activation of caspase 9 by released cytochrome *c* is the most upstream ATP-dependent step in type II cells. These observations also confirm the existence of two pathways for Fas-mediated apoptotic signal transduction and suggest that the Apaf-1 (Ced-4 homologue) system for caspase 9 activation operates in an ATP-dependent manner *in vivo*.

INTRODUCTION

Apoptosis accounts for most physiological cell death and is defined by characteristic morphological changes, including fragmentation of nuclei with condensation of chromatin, condensation of the cytoplasm, and formation of apoptotic bodies (1, 2). Apoptosis is dependent on tightly regulated cell death signaling pathways. The cysteine protease encoded by *Caenorhabditis elegans ced*-3 and its mammalian homologues, designated as caspases, seem to play a key role in driving apoptosis (3, 4). Caspases are synthesized as inactive proforms and are activated by proteolytic cleavage at the COOH-terminal side of specific aspartic acid residues to form heterotetrameric complexes composed of two each of the large and small cleavage products on apoptosis-inducing treatment (3, 4). Caspases can be classified into two groups (4): initiator caspases represented by caspases 8 and 9 having a long prodomain at their amino-terminal portion that can be self-cleaved on oligomerization (5–7); and effector caspases represented by caspases 3, 6 and 7 that have a relatively short prodomain and can be activated by upstream initiator caspases or by activated effector caspases. Thus, caspases form a protease cascade that transmits apoptotic signals (3, 4).

Recent studies have suggested that mitochondria play an essential role in apoptotic signal transduction (8, 9). Various apoptotic stimuli have been shown to induce mitochondrial changes (10–11), resulting in release of apoptogenic factors such as mitochondrial cytochrome *c* (12–14) and apoptosis-inducing factor (15, 16) into the cytoplasm, which are observed in the early phase of apoptosis. The release of these proteins is prevented by mitochondrial antiapoptotic proteins, Bcl-2 (17–19) and Bcl-x_L (20), for which the *C. elegans* homologue is Ced-9 (21), via an unknown mechanism (8).

Biochemical studies have revealed that cytochrome *c* interacts with a cytoplasmic factor called Apaf-1 (22), the mammalian homologue of the *C. elegans* death regulator Ced-4, and the complex recruits procaspase 9 (Apaf-3) (23) to induce its oligomerization (7). The formation of the ternary complex has been shown to proceed in a dATP (or ATP)-dependent manner *in vitro* (23). Procaspase 9 is autoactivated on oligomerization, which in turn activates caspase 3 (23). In addition to the prevention of release of mitochondrial apoptogenic factors to the cytoplasm, Bcl-2/Bcl-x_L has also been shown to inhibit caspase 9 activation via forming ternary complex called apoptosome, comprising Bcl-2/Bcl-x_L-Apaf-1-procaspase 9 (24, 25). The activation of effector caspases such as caspase 3 results in proteolytic cleavage of various cellular substrates (3, 4), including DFF45(ICAD) (26, 27), which is an inhibitor of CAD (DFF40), a DNase required for oligonucleosomal DNA fragmentation (28, 29).

Apoptotic signals triggered by Fas (Apo1/CD95) antigen, the death receptor, were recently reported to be transmitted by two pathways depending on the type of cell (30). In type I cells, Fas stimulation activates caspase 8 by rapid formation of the DISC[3] (31), followed by activation of caspase 3, and apoptosis as well as the activation of these caspases are not blocked by Bcl-2. In type II cells, DISC formation is less prominent and mitochondrial changes as assessed by mitochondrial membrane potential ($\Delta\Psi m$) precede the activation of caspases 8 and 3, with overexpression of Bcl-2 inhibiting both apoptosis and activation of these caspases. Thus, death signals in Fas-mediated apoptosis are transmitted by either mitochondria-dependent or -independent pathways. Fas-mediated apoptosis involving the mitochondria-dependent pathway seems to share the machinery for transmission of apoptotic signals with other cell death stimuli, including calcium ionophore, γ-irradiation, chemotherapy drugs, and reactive oxygen species (8, 9).

We and others have recently shown that depletion of the intracellular ATP completely blocks Fas-mediated apoptosis (32, 33) and that ATP-dependent steps exist both upstream and downstream of caspase 3-like protease activation during apoptotic signal transduction in Jurkat and HeLa cells (32), both of which are classified as type II cells. We also suggested that the ATP level is a determinant of cell death by apoptosis or necrosis (32, 33). Because apoptotic death signals are probably transmitted from the cytoplasm to the nucleus, one of the ATP-dependent steps functioning downstream of the activation of caspase 3 might be an active nuclear transport mechanism, which requires ATP hydrolysis (34, 35). Indeed, we have previously shown that active nuclear transport is essential for apoptotic changes of the nucleus to occur (36).

In the present study, we examined the ATP-dependent steps of Fas-mediated apoptotic signal transduction in type I cells, which are likely to be specific for death receptor-mediated apoptosis. We also

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[3] The abbreviations used are: DISC, death-inducing signaling complex; RITC, rhodamine B isothiocyanate; RT, room temperature.

studied ATP-dependent steps in type II cells, which involve the mitochondria and, therefore, are likely to be related to apoptosis in general. Our results indicated that the ATP-dependent step(s) of Fas-mediated apoptosis in type I cells are located downstream of the activation of caspase 3, whereas the activation of caspase 9 is the most upstream ATP-dependent step in type II cells.

MATERIALS AND METHODS

Cell Lines and Culture. SKW6.4 cells, a human B lymphoblastoid cell line, and Jurkat cells, a human T cell line, were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 2 mM glutamate. Depletion of intracellular ATP was achieved as described (32) by incubating cells for 1 h in the presence of 10 μM oligomycin in glucose-free DMEM (Life Technologies, Inc.) supplemented with 50 mM malic acid, 2 mM glutamate, 1 mM sodium pyruvate, 10 mM HEPES/Na^+ (pH 7.4), 0.05 mM 2-mercaptoethanol, and 10% dialyzed fetal bovine serum (Life Technologies, Inc.). Under these conditions, oligomycin inhibits mitochondrial F_0F_1-ATPase (37) and the depletion of glucose halts glycolysis, so that no ATP-producing machinery operates.

Death-inducing Treatments. SKW6.4 and Jurkat cells (10^6 cells/ml) with or without 1 h of pretreatment to deplete intracellular ATP, as described above, were incubated with 0.3 μg/ml and 0.2 μg/ml, respectively, of the agonistic antihuman Fas monoclonal antibody CH-11 (MBL, Nagoya, Japan) for various periods. The concentration of anti-Fas antibody was determined to induce apoptosis in SKW6.4 and Jurkat cells in a similar kinetics (see Fig. 2). Apoptotic and necrotic cells were identified using fluorescence microscopy with Hoechst 33342 and propidium iodide, as described previously (38). For quantitative analysis, more than 1000 cells were counted.

Detection of Cleavage of Procaspases and DFF45 (ICAD). SKW6.4 and Jurkat cells were treated with antihuman Fas antibody, as described above. At the indicated times, cells were harvested and lysed in SDS-sample buffer [0.04 M Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 5% glycerol, and 0.05% BPB]. An aliquot of the lysate (1×10^5 cells) was then analyzed by Western blotting to detect cleavage of procaspases using antihuman caspase 3-p11 goat polyclonal antibody (K-19; Santa Cruz Biotechnology), antihuman caspase 8 mouse monoclonal antibody (5F7; MBL), and antihuman caspase 9 rabbit polyclonal antibody raised against recombinant GST-caspase 9 fusion protein in our laboratory. Immunoreactive bands were visualized using horseradish peroxidase-conjugated species-specific secondary antibodies and enhanced chemiluminescence detection reagents (Amersham Corp.). Antihuman DFF45 mouse monoclonal antibody (6B8; MBL) was used for analysis of DFF45 (ICAD) cleavage to evaluate caspase activation.

Immunohistochemical Analysis of Subcellular Localization of Cytochrome *c*. SKW6.4 and Jurkat cells were treated with anti-Fas antibody under various conditions for 2 h. Cells were then harvested, washed with PBS, and fixed with 7% formaldehyde in PBS for 10 min at RT. Fixed cells (10^5 cells) were applied to slide glasses, dried, washed twice with PBS, and permeabilized with 0.5% Triton X-100 in PBS for 10 min at RT. After washing with PBS, the cells were incubated with antibovine cytochrome *c* mouse monoclonal antibody (7H8; Pharmingen) and antibovine F_1-ATPase rabbit polyclonal antibody, which recognize human cytochrome *c* and human F_1-ATPase, respectively, at 4°C for 24 h. After washing with PBS, cells were then incubated with RITC-conjugated antimouse IgG antibody and FITC-conjugated antirabbit IgG antibody for 15 min at RT. Cells were washed with PBS and then observed under a fluorescent microscope (BX50; Olympus, Tokyo, Japan) with excitation at 480 nm for FITC and at 530 nm for RITC.

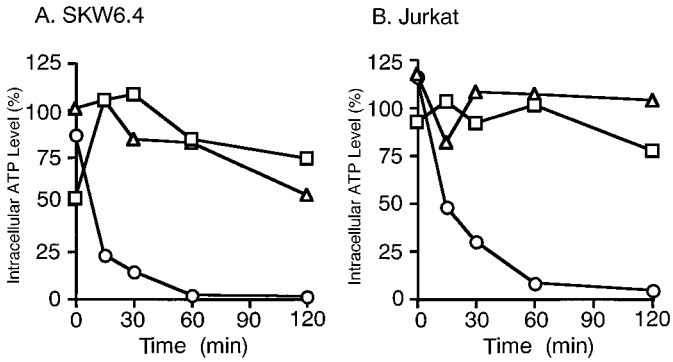


Fig. 1. Depletion of intracellular ATP. Intracellular ATP level of SKW6.4 cells (*A*) and Jurkat cells (*B*) were determined by the luciferin-luciferase (Sigma Chemical Co., St. Louis, MO) method (49) after cells were incubated for the indicated periods with 10 μM oligomycin in glucose-free DMEM (○), with 10 μM oligomycin in 0.35% glucose-containing DMEM (△) and without oligomycin in glucose-free DMEM (□). ATP level of cells cultured under normal conditions was regarded as 100%.

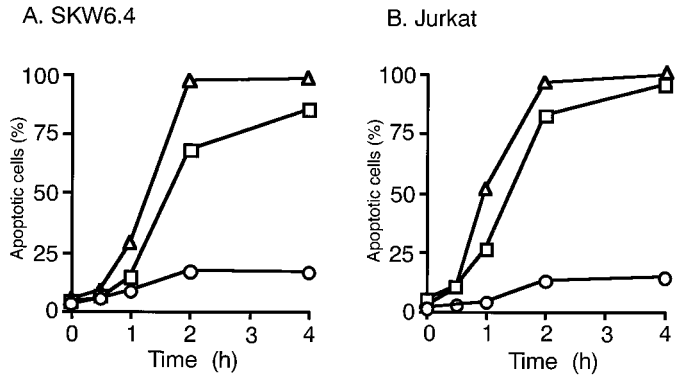


Fig. 2. Inhibition of apoptotic nuclear changes by depletion of intracellular ATP. SKW6.4 (*A*) or Jurkat cells (*B*) were preincubated under ATP-replete or ATP-depleted conditions for 1 h, as described below, and then were cultured with 0.3 μg/ml or 0.2 μg/ml, respectively, of anti-Fas monoclonal antibody (CH-11). The extent of cell death was assessed by counting viable, necrotic, and apoptotic cells under a fluorescent microscope after double-staining of the cells with Hoechst 33342 and propidium iodide for 3 min. More than 1000 cells were counted in each experiment. ○, incubation with 10 μM oligomycin in glucose-free DMEM (ATP-depleted); △, incubation with 10 μM oligomycin in 0.35% glucose-containing DMEM (ATP-replete); □, incubation in glucose-free DMEM without the addition of oligomycin (ATP-replete).

Biochemical Subcellular Fractionation of Apoptotic Cells. SKW6.4 and Jurkat cells were treated with anti-Fas antibody, as described above. At the indicated times, cells were harvested, washed with PBS and incubated with 10 μM digitonin (Sigma Chemical Co.) in mitochondrial isolation buffer [0.3 M mannitol, 0.2 mM EDTA/K, 0.1% fatty acid-free BSA, 10 mM Hepes-KOH (pH 7.4), with 1 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin] at 10^7 cells/ml at 37°C for 3 min. This treatment resulted in disruption of the cell membrane, but not the mitochondrial membrane. Conditions were chosen to achieve only 50% cell lysis, which was assessed by the release of lactate dehydrogenase (data not shown), to minimize damage to the mitochondria during fractionation. After centrifugation at 3,000 *rpm* for 2 min at 4°C, aliquot of the supernatant (cytoplasmic fraction) and the precipitate (containing mitochondria) corresponding to 10^5 cells each were analyzed by Western blotting using antibovine cytochrome *c* mouse monoclonal antibody (7H8; Pharmingen). Immunoreactive bands were visualized using horseradish peroxidase-conjugated antimouse IgG antibody and enhanced chemiluminescence detection reagents.

RESULTS

Apoptotic Nuclear Changes under ATP-depleted Conditions. To elucidate the ATP-dependent mechanisms of apoptosis, we selected SKW6.4 and Jurkat cell lines as representatives of type I and type II cells, respectively, in which Fas-mediated apoptotic signals are transmitted via different pathways (30). Intracellular ATP levels were manipulated, as described previously (32), by incubating cells in glucose-free medium with 10 μM oligomycin (an inhibitor of mitochondrial F_0F_1-ATPase) to block the production of ATP by both glycolysis and oxidative phosphorylation (37). Under these conditions, intracellular ATP became undetectable within 60 min in both SKW6.4 and Jurkat cells, whereas the addition of glucose or omission of oligomycin maintained the level of ATP (Fig. 1). ATP depletion did not alter Fas-antigen expression on the cell surface (32).

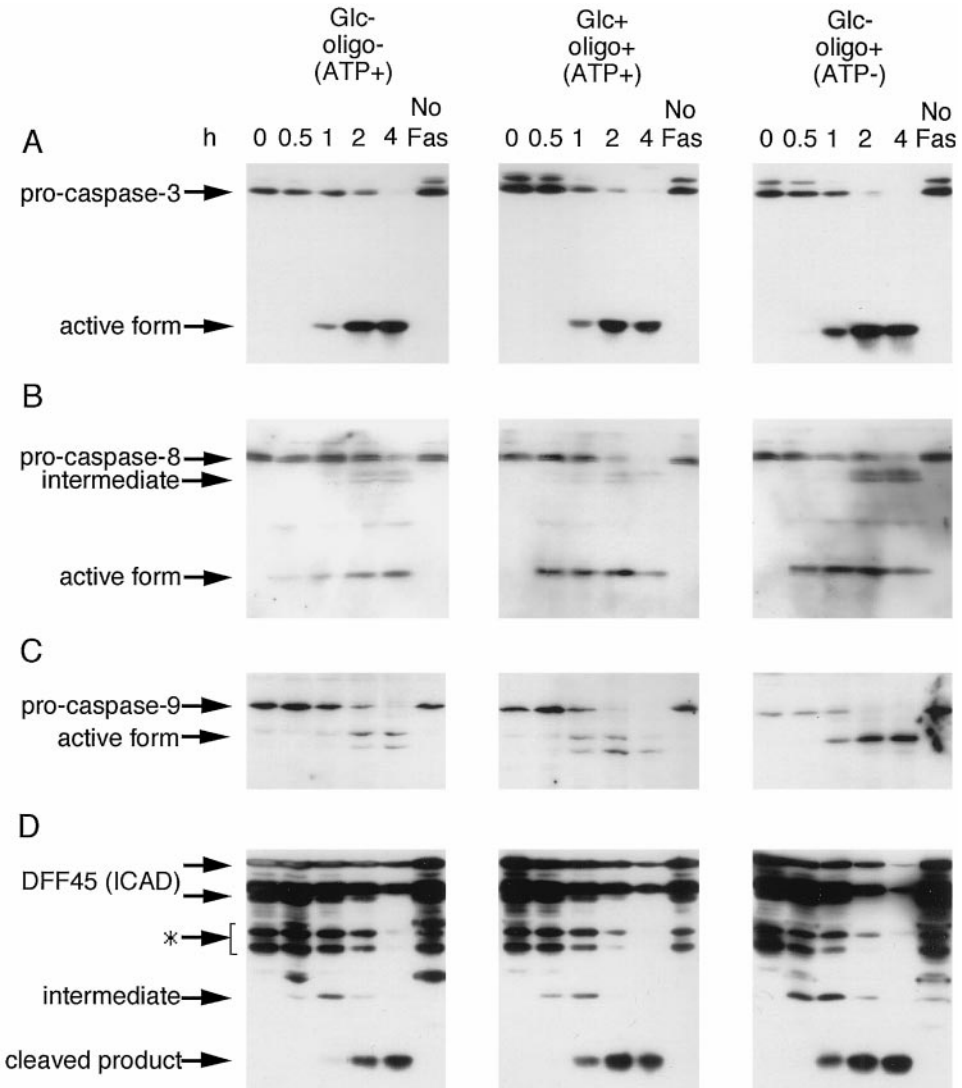


Fig. 3. Fas-mediated cleavage of procaspases and DFF45 (ICAD) in SKW6.4 cells under intracellular ATP-depleted conditions. SKW6.4 cells were treated for the indicated periods, as described in the legend to Fig. 2. Cleavage of procaspases 3 (*A*), 8 (*B*), and 9 (*C*), and also that of DFF45 (ICAD) (*D*), were analyzed by Western blotting as described in "Materials and Methods." Cell treatments and the positions of procaspases or DFF45 (ICAD) and their cleaved products are also shown. *No Fas*, cells incubated for 4 h without Fas stimulation under the same conditions. *, degraded forms, short isoforms, or homologues of DFF45 (ICAD).

First we examined whether Fas-mediated signals reached to nucleus under the ATP-depleted conditions to induce apoptotic nuclear changes, including chromatin condensation and nuclear fragmentation, which seem likely to be the last steps of apoptotic signal transduction. When Jurkat cells were treated with an agonistic anti-Fas monoclonal antibody under ATP-depleted conditions, only a few apoptotic cells were found (Fig. 2*B*), whereas many apoptotic cells appeared when ATP was supplied by glycolysis after the addition of glucose or supplied by the mitochondria after omitting oligomycin from the glucose-free medium (Fig. 2*B*), confirming our previous observations (32). Similarly, ATP depletion greatly reduced the induction of apoptotic nuclear changes in SKW6.4 cells (Fig. 2*A*). The large number of apoptotic cells in cultures where ATP was supplied either by the mitochondria or by glycolysis (Fig. 2) argues against the possibility that apoptosis might be glucose-dependent or oligomycin-sensitive, but not ATP-dependent. The number of necrotic cells was not increased significantly during incubation for 4 h under ATP-depleted conditions, but longer incubation increased the occurrence of necrosis (data not shown). From these results, we concluded that Fas-mediated apoptotic nuclear changes were ATP-dependent in both type I and type II cells.

Activation of Caspases in SKW6.4 Cells under ATP-depleted Conditions. To define how far Fas-mediated apoptotic signal transduction could proceed under ATP-depleted conditions, we examined whether or not various caspases were activated by analyzing the cleavage of procaspases. We selected caspases 3, 8, and 9, because the activation of caspase 3 seems likely to be one of the last steps in the caspase cascade, whereas activation of caspase 8 by DISC and activation of caspase 9 by Apaf-1 and cytochrome *c* are likely to be the initial steps of the caspase cascade in type I and type II cells, respectively.

When SKW6.4 cells were treated with agonistic anti-Fas antibody under ATP-depleted conditions, procaspase 3 was cleaved similarly to under ATP-replete conditions (Fig. 3*A*), indicating that activation of caspase 3 by Fas stimulation did not require the presence of intracellular ATP in type I cells. As expected from the ATP-independent activation of caspase 3, upstream caspase 8 was rapidly cleaved irrespective of the intracellular ATP level (Fig. 3*B*), indicating that activation of caspase 8 by DISC was independent of ATP. As suggested by the previous observation that caspase 9 was probably activated by caspase 3 in type I cells (30), caspase 9 was also activated irrespective of the intracellular ATP level (Fig. 3*C*). Consistently, the cleavage of DFF45 (ICAD), an inhibitor of CAD (DFF40) and one of the substrates for caspase 3, was not affected by depletion of intracellular ATP in SKW6.4 cells (Fig. 3*D*). These results indicated that activation of caspases including caspase 8 was not ATP-dependent in SKW6.4 cells, in which rapid DISC formation has been reported (30),

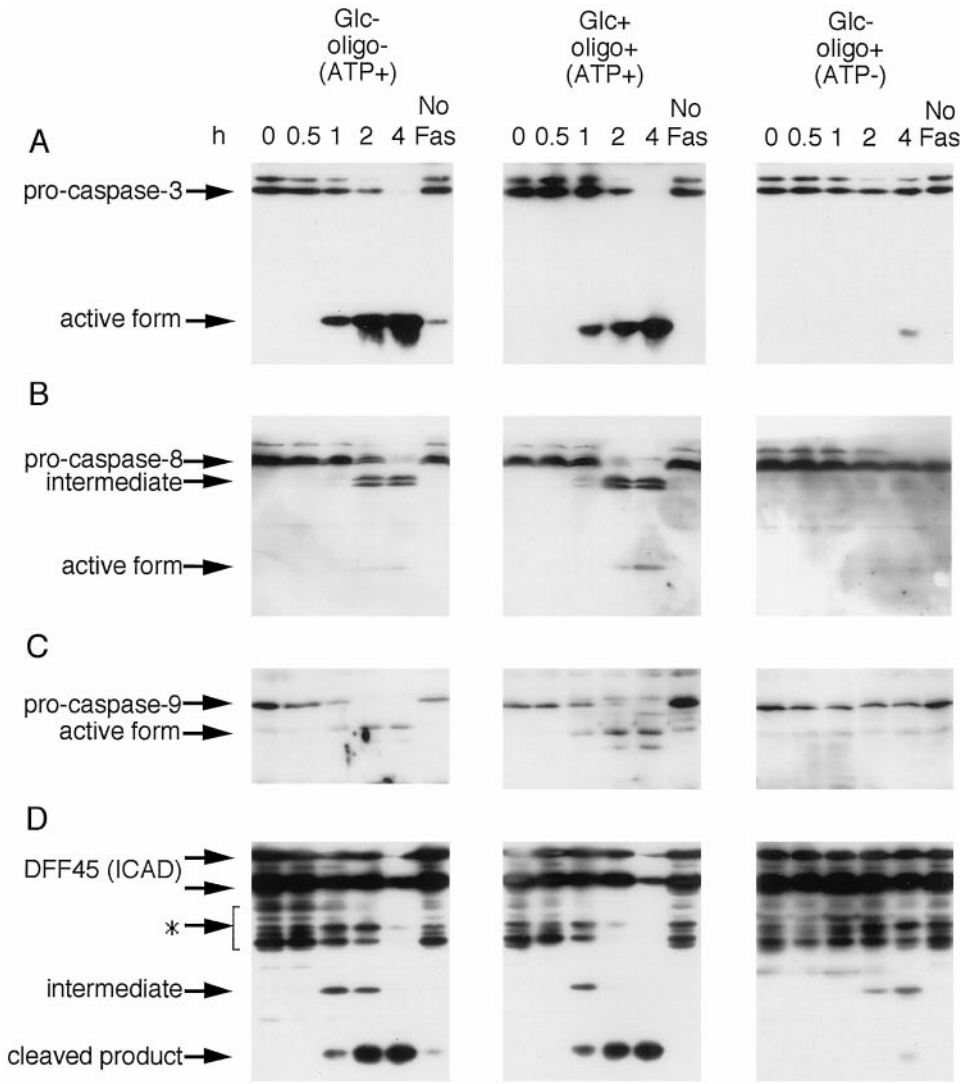


Fig. 4. Inhibition of Fas-mediated cleavage of procaspases and DFF45 (ICAD) in Jurkat cells by depletion of intracellular ATP. Jurkat cells were treated for the indicated periods, as described in the legend to Fig. 2. Cleavage of procaspase 3 (*A*), 8 (*B*), and 9 (*C*), and also that of DFF45 (ICAD) (*D*), were analyzed by Western blotting, as described in "Materials and Methods." Cell treatments and the positions of procaspases or DFF45 (ICAD) and their cleaved products are also shown. For definitions of *No Fas* and ∗, see Fig. 3 legend.

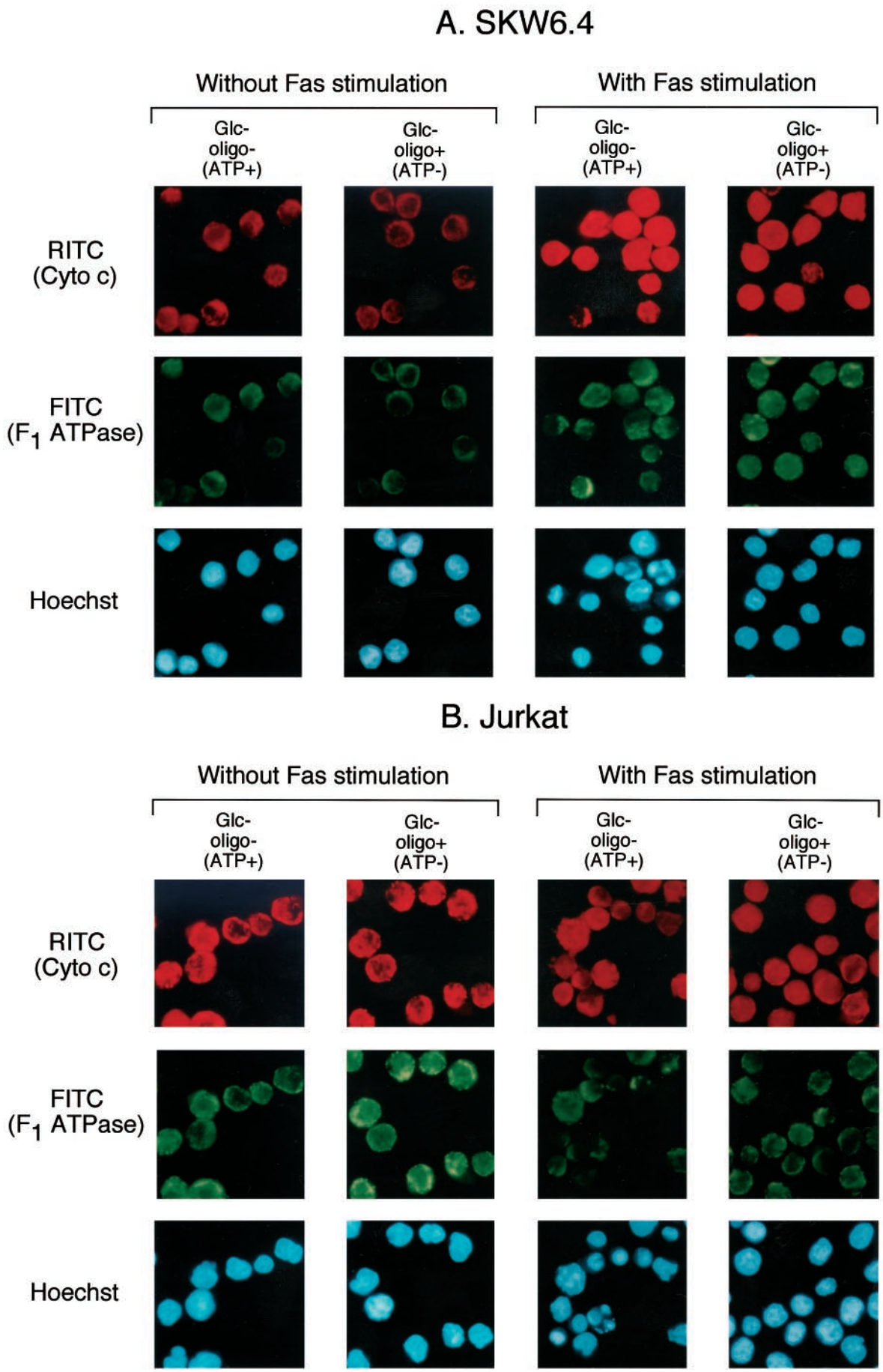
and that ATP-dependent steps could only exist downstream of the activation of caspase 3 in these cells.

No Activation of Caspases in Jurkat Cells under ATP-depleted Conditions. We next examined whether the activation of caspases was affected by depleting intracellular ATP in Jurkat cells. When Jurkat cells were treated with anti-Fas antibody under ATP-replete conditions, procaspase 9 was cleaved. However, the cleavage of this procaspase was inhibited by depletion of intracellular ATP (Fig. 4*C*). As expected from this result, cleavage of the downstream procaspase, procaspase 3, was also inhibited by ATP depletion (Fig. 4*A*). The results were consistent with our previous observation that an increase in caspase 3 activity assessed by hydrolysis of the specific substrate DEVD-MCA was inhibited by intracellular ATP depletion in Jurkat and HeLa cells (32). Cleavage of DFF45 (ICAD) and caspase 8, which was also suggested to be catalyzed by activated caspases in type II cells, was also inhibited by intracellular ATP depletion in Jurkat cells (Fig. 4, *B* and *D*). The results indicated that ATP-dependent steps were present upstream of the activation of caspase 9 in Jurkat cells.

Release of Cytochrome *c* under ATP-depleted Conditions. In many apoptotic systems, signals are transmitted to the mitochondria to induce the release of cytochrome *c* into the cytoplasm, which then induces the cleavage/activation of procaspase 9 in cooperation with cytoplasmic Apaf-1 (22, 23). Although the Apaf system is likely to operate in type II cells (30), the immediate downstream step (activation of caspase 9) was inhibited by ATP depletion (Fig. 4*C*). To examine the possibility that ATP depletion affects cytochrome *c* release, we determined the subcellular localization of cytochrome *c* after treatment with anti-Fas antibody by fluorescent microscopy and by biochemical fractionation of cells.

Fluorescent microscopy revealed that cytochrome *c* was colocalized with F_1-ATPase (a protein found on the mitochondrial inner membrane) in a compact and granular fashion in the absence of Fas stimulation, regardless of the intracellular ATP level in both SKW6.4 and Jurkat cells (Fig. 5), indicating the mitochondrial localization of cytochrome *c*. When cells were treated with anti-Fas antibody under ATP-replete conditions, fluorescent signals for cytochrome *c* became diffuse compared with those for F_1-ATPase, which remained granular, indicating the release of cytochrome *c* into the cytoplasm (Fig. 5). Even in the absence of intracellular ATP, cytochrome *c* became diffuse after Fas treatment of both SKW6.4 and Jurkat cells (Fig. 5), suggesting that Fas-mediated cytochrome *c* release was not affected by ATP depletion in these cell types.

Biochemical fractionation was carefully performed after permeabilizing cells with digitonin, a detergent relatively specific for the plasma membrane, under conditions allowing the recovery of active mitochondria. The extent of cell lysis was limited to about 50%, as described in "Materials and Methods," to minimize damage to the



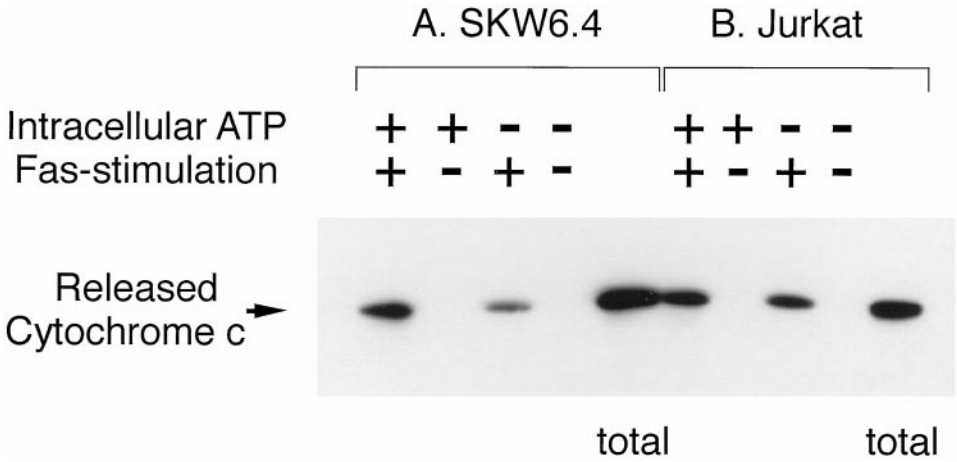


Fig. 6. Fas-mediated release of cytochrome *c* analyzed by fractionation. SKW6.4 (*A*) or Jurkat (*B*) cells were treated as described in the legend to Fig. 2, and the cytoplasmic and membrane fractions were recovered as described in "Materials and Methods." Then the presence of cytochrome *c* in the cytoplasmic fraction was analyzed by Western blotting using anti-cytochrome *c* antibody. Cell treatments and the position of cytochrome *c* are also shown.

mitochondria during fractionation. In both SKW6.4 and Jurkat cells, a significant amount of cytochrome *c* was recovered in the cytoplasmic fraction after Fas treatment, regardless of the intracellular ATP level (Fig. 6), consistent with the results of immunostaining shown in Fig. 5.

DISCUSSION

Apoptosis is characterized by morphological changes, including fragmentation of the nucleus with condensation of chromatin. Recent studies have revealed that the apoptotic nuclear changes induced by various death treatments are completely blocked by depleting the intracellular ATP (32, 33). One of the ATP-dependent steps located downstream of caspase 3 activation (32) should be active nuclear transport, which has been shown to be essential for apoptosis in response to various stimuli (36). We have also shown that signaling pathways upstream of caspase 3 activation include ATP-dependent steps, at least in some apoptotic systems using dexamethasone, calcium ionophore, and VP-16 (32).

To investigate the apoptotic signal transduction, we analyzed the ATP-dependence of the well-characterized Fas-mediated process of apoptosis, which has recently been reported to occur via two distinct signaling pathways depending on cell type (30). Type I cells are characterized by rapid formation of DISC and activation of caspase 8 after Fas stimulation, followed by activation of caspase 3 and release of cytochrome *c* from the mitochondria into the cytoplasm. In these cells, Fas-mediated apoptosis is not inhibited by overexpression of Bcl-2, suggesting that mitochondrial dysfunction and cytochrome *c* release are not essential for Fas-mediated apoptosis, but are rather a consequence of caspase activation. On the other hand, type II cells show little detectable DISC formation and relatively slower activation of caspases than type I cells. Fas-mediated apoptosis of type II cells is inhibited by Bcl-2 overexpression and has been suggested to involve the mitochondria in its signal transduction pathway and to share its main machinery with other apoptotic stimuli.

In this study, we showed that caspase 3 was fully activated independent of intracellular ATP in type I cells treated with anti-Fas antibody and the ATP-dependent step was downstream of the activation of caspase 3. On the other hand, the activation of caspase 9, which occurs just downstream of the release of cytochrome *c*, seemed to be the most upstream ATP-dependent step in type II cells (Fig. 7). Because apoptosis other than that induced by death receptor stimulation is generally mediated by mitochondrial changes, which are prevented by overexpression of Bcl-2 and Bcl-x_L (8–11), the activation of caspase 9 is suggested to be one of the major common ATP-dependent steps in apoptosis. In both type I and II cells, active nuclear transport should be one of the common downstream ATP-dependent steps in apoptotic signal transduction. It is possible that a machinery of nuclear changes requires ATP, as well. These observations on ATP dependence also help to define the existence of two distinct pathways for Fas-mediated apoptotic signal transduction.

In vitro analysis using isolated nuclei has indicated that cytochrome *c* can interact with Apaf-1 (22), and that the complex recruits procaspase-9 to induce its oligomerization (23). Procaspase 9 has been suggested to be auto-activated on oligomerization (7), in turn activating caspase 3. Because the oligomerization step has been shown to proceed in a dATP (or ATP)-dependent manner *in vitro* (23), the ATP-dependent step between the release of cytochrome *c* and the activation of caspase 9 observed here should be the oligomerization of procaspase 9 by the cytochrome *c*/Apaf-1 complex. Thus, our results strongly suggest that the Apaf system activating caspase 9 actually operates in an ATP-dependent manner *in vivo*.

Both the Apaf and DISC systems involve the recruitment of precursor forms of the initiator caspases via their CARD and death effector domains, respectively, to induce activation of the caspases. Despite this similarity at the entry step into the caspase cascade, the DISC system was shown to be ATP-independent and the Apaf system was shown to be ATP-dependent. The physiological significance of the ATP dependency of the Apaf system is now unclear, but it might distinguish the physiological release of cytochrome *c*, which should lead to apoptosis, from accidental release which might be induced by necrosis where intracellular ATP is often lost through disruption of the cell membrane. Death receptor-mediated cell death seems to be a special form of apoptosis, in a sense that it bypasses the mitochondria and directly activates the caspase cascade. The significance of the ATP-dependence of the Apaf system should be elucidated by further investigations.

Fig. 5. Immunofluorescence analysis of Fas-mediated release of cytochrome *c*. SKW6.4 (*A*) or Jurkat (*B*) cells were treated as described in the legend to Fig. 2. Cells were then fixed and immunostained with anti-cytochrome *c* antibody and anti-F_1-ATPase antibody, as described in "Materials and Methods." Cells were visualized under a fluorescent microscope with the aid of RITC- or FITC-labeled secondary antibodies. Shown are representative fluorescence micrographs of cells taken from the same field, with excitation at 530 nm for RITC, 480 nm for FITC, and 360 nm for Hoechst 33342. Cell treatments are also shown.

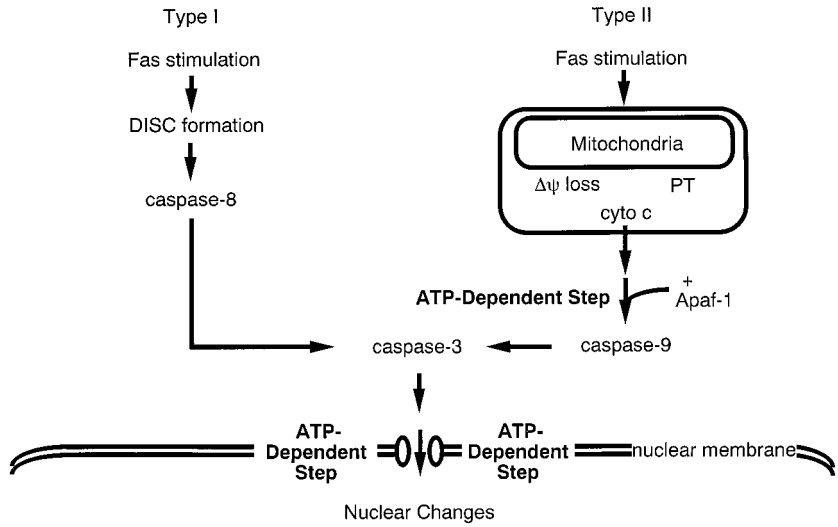


Fig. 7. ATP-dependent steps of the Fas-mediated apoptotic signal transduction pathway in type I and II cells. Diagram of the Fas-mediated apoptotic signal transduction pathways in type I and II cells is shown. The ATP-dependent steps of Fas-mediated apoptotic signal transduction in type I cells are only located downstream of caspase 3 activation, probably including active nuclear transport and/or a machinery of nuclear changes. The activation of caspase 9 by released cytochrome *c* is the most upstream ATP-dependent step in type II cells, and like in type I cells, ATP-dependent steps are also located downstream of caspase 3 activation in these cells.

It was recently suggested that proapoptotic Bcl-2 family members, including Bax and Bid, might transmit apoptotic signals to the mitochondria, based on the observations that Bax and caspase-cleaved Bid are transferred to the mitochondria from the cytoplasm in response to apoptosis-inducing stimuli (39–43) and can induce cytochrome *c* release in isolated mitochondria (42–45). The apoptosis induced by overexpression of Bax has been reported to be prevented through inhibition of F_0F_1-ATPase by oligomycin (46). As described here and in previous studies, inhibition of F_0F_1-ATPase by oligomycin does not necessarily prevent various types of apoptosis, including Fas-mediated apoptosis (32, 33, 47). Assuming that all proapoptotic Bcl-2 family members act on the mitochondria in the same way as Bax, apoptotic signal transmission to the mitochondria in oligomycin-insensitive apoptosis is likely to be mediated by molecules other than proapoptotic Bcl-2 family members that do not require functional F_0F_1-ATPase *in vivo*. Thus, although mitochondrial changes induced by proapoptotic Bcl-2 family members might be involved in apoptotic signal transduction in certain circumstances, this does not seem to sufficiently explain the general mechanism of apoptosis *in vivo*. Further investigations on signal transmission to the mitochondria by methods that do not depend on F_0F_1-ATPase is necessary.

A recent study showed that Fas-mediated apoptosis of Jurkat cells does not require intracellular ATP, whereas apoptosis induced by other stimuli requires ATP, suggesting that Fas-mediated apoptosis and that caused by other stimuli can be classified by their ATP requirement (48). However, the culture medium used contained 1% nondialyzed serum, which may have a trace amount of glucose to support glycolysis. Because Fas-mediated apoptosis of Jurkat cells requires much less intracellular ATP than apoptosis induced by other stimuli (33), the condition used by Ferrari *et al.* (48) might provide sufficient ATP to transmit Fas-mediated apoptotic signals, but not to support apoptosis induced by chemotherapy drugs.

In conclusion, we defined the most upstream ATP-dependent step of apoptotic signal transduction as the activation of caspase 9 in the Apaf system, except for Fas-mediated apoptosis of type I cells, although we cannot exclude the possible involvement of kinases with a very low Km to ATP that might function upstream of the activation of caspase 9.

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