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Cell Death-Associated Translocation of Plasma Membrane Components Induced by CTL

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In the very early stages of target cell apoptosis induced by CTL, we found that fluorescence of labeling probes of the target plasma membrane, such as N-(3-triethylammoniumpropyl)-4-(p-dibutylaminostyryl)pyridinium dibromide (FM1-43), was translocated into intracellular membrane structures including nuclear envelope and mitochondria. This translocation was associated with the execution of CTL-mediated killing, because neither the CTL-target conjugation alone nor the binding of noncytotoxic Th2 clone with target cell was sufficient to provoke the process. Although FM1-43 translocation was observed in perforin-mediated cytotoxicity, examinations with several other dyes failed to detect the evidence for membrane damages that may cause influx of the dye. Moreover, the translocation was also observed in Fas-dependent apoptosis. These data indicate that the translocation precedes the damage of plasma membrane and intracellular organelles in the course of apoptotic cell death and may represent the existence of a membrane trafficking that mediates the translocation of plasma membrane components in the early onset of apoptotic cell death. The Journal of Immunology, 2000, 164: 4641–4648.

Apoptosis is essential for ontogenetic development as well as remodeling of normal adult tissues. Dysregulation of the apoptotic response may lead to the development of cancer, and autoimmune disease as well as degenerative disorders. Operationally, apoptosis is initiated by “death receptors” (TNF receptor, Fas, DR3, DR4, and DR5), by p53-dependent and -independent cellular stress pathways (genotoxic pathways) that induce permeability transition in mitochondria (Mt) and release of cytochrome c, and by the secretion of granules that contain perforin and granzymes from cytotoxic cells such as CTL. As a consequence, a family of phylogenetically conserved cysteine proteases (caspases) are activated (1). The caspases are divided into apical (caspase-2, -8, -9, and -10) and executioner subsets (caspase-3, -6, and -7). Ligation of a death receptor or cellular stress results in activation of specific apical members. Regardless of the stimulus, all apical caspases appear to converge at caspase-3, after which caspase-6 and -7 become activated (1). These executioner caspases then cleave regulatory and structural proteins that produce an orderly dismantling and clearance of the dying cell. Caspase-3 is also implicated in the activation of apoptosis-specific DNase by cleaving its inhibitor (2).

While much attention to the “death receptor” and genotoxic pathways have lead to remarkable advances in the understanding to these signaling cascades, the mechanism that underlies apoptosis via the granule-dependent pathway, another pathway in killer cell-mediated cytotoxicity (3, 4), remains unclear. It is generally accepted that granzymes are injected into the cytosol of the target cell through pores formed by polymerized perforin, polyperforin. However, recent reports showed sublytic doses of perforin suffice to deliver granzymes to the cytosol (5–8), suggesting that perforin may not merely function as a perforating agent. Coupled with the recent observation that granzyme B appears to be specifically endocytosed in a receptor-dependent manner (5), the hypothesis has been presented that membrane-associated perforin and the granzyme are internalized coincidentally, and the endosomolytic action of perforin leads to the release of granzyme B to the cytosol where the protease activates the caspase cascade (9).

It is known that apoptotic cells express phosphatidylserine (PS) on the surface that usually resides in the inner layer of plasma membrane lipid bilayer, implying that perturbation of plasma membrane components takes place in the process (10, 11). Nevertheless, the mechanisms underlying the alteration in membrane trafficking during apoptosis are totally unknown. During an analysis of CTL degranulation with various fluorochromes, we unexpectedly observed that the fluorescence of N-(3-triethylammoniumpropyl)-4-(p-dibutylaminostyryl)pyridinium dibromide (FM1-43), a cell-surface labeling probe, was translocated into the intracellular membrane structures of target cell. FM1-43, a fluorogenic styryl dye, has been extensively used to visualize exocytic and endocytic
activity (12–15). The possibility was considered that internalization of FM1-43 was a consequence of the “lethal hit” delivered by the CTL. A series of experiments were performed to determine the mechanism responsible for translocation of the dye and to learn whether the process accompanied granule-mediated and Fas-dependent apoptosis.

Materials and Methods
Ab and reagents
Anti-Fas mAb, Jo2 (16), was purchased from PharMingen (San Diego, CA). Another murine Fas-specific rat mAb, RMF-2, was a generously provided by Dr. Shin Yonehara (Kyoto University, Kyoto, Japan) and biotinylated in our laboratory. Anti-murine FcR mAb, 2,4G2, was used in a concentrated form of culture supernatant. A murine CD8-specific rat mAb, 2.43, was used as a negative control for flow cytometry. FITC-streptavidin and FITC-labeled rabbit anti-rat Ig, which is not cross-reactive to murine Ig, were prepared in our laboratory. Anti-murine CD3 mAb 145-2C11 (2C11) (17) was purified from culture supernatant or ascites fluid with a protein A-Sepharose affinity column. Purified human perforin was prepared from YT granules as previously described (5).

Fluorescent probes FM1-43 (13), 8-hydroxy-pyrene-1,3,6-trisulfonic acid (HPTS), 1′,1′-dioctadecyl-3,3,3′,3′-tetratramethylindocarbocyanine perchlorate (DiIC12) (18), MitoTracker Red CMXRos, 5-carboxyfluorescein diacetate acetoxyethyl ester (CFDA-AM), dihydroethidium (DHE), and 6-carboxyfluorescein (6-CF) are products of Molecular Probes (Eugene, OR). FITC-conjugated succinylated Con A (FITC-succ.ConA), Acridine Orange, quinacrine, and FITC-dextran were purchased from Sigma (St. Louis, MO). Texas Red-conjugated cytochrome c (TXR-cyt.c) was prepared in our laboratory. Acetyl-Asp-Glu-Val-Asp-(4-methylcoumaryl-7-amide) (Ac-DEVD-MCA) is a product of the Peptide Institute (Osaka, Japan).

Cells
Murine B lymphoma A20.2J (H-2d) and a mast cell line PT18 (H-2b) (19), generously provided by Dr. Chisei Ra (Juntendo University School of Medicine), were maintained by in vitro culture. A variant of A20.2J, 1L1-A20-Jo2R, was isolated from transfectant with a A20.2J-derived cDNA clone. An H-2d-specific CD8− CTL clone OE4 was maintained as previously described (20). A perforin-deficient H-2d-specific CD8+ CTL line, P0K, was established by gene targeting (3). H-2d-specific Fas ligand (FasL)-expressing A20.2J clones, 1L1-A20-Jo2R was measured by cleavage of a tetra-peptide substrate Ac-DEVD-MCA, 0.05 M NaCl, 2.5 mM DTT, 10 mM HEPES, pH 7.5. The 50-μl aliquot of lysate was mixed with 150 μl substrate solution (40 μM Ac-DEVD-MCA, 0.05 M NaCl, 2.5 mM DTT, 10 μM HEPES, pH 7.5) and incubated at 37°C for 2 h. Fluorescence of released 7-amino-4-methylcoumarin was measured by FluoroScan fluorescence microplate reader with 355 nm excitation and 460 nm emission filter set. The activity was expressed as the F.I. with arbitrary units.

Flow cytometric analysis of cell-surface protein expression and CTL-target conjugate formation
Cell-surface expression of Fas and FcR was analyzed by binding of rat anti-mouse Fas mAb, RMF-2, and of rat anti-mouse FcR mAb, 2,4G2, respectively. Rat anti-mouse CD8 mAb, 2.43, was used as a negative control for FcR staining. Cells were analyzed by flow cytometry with FACSscan (Becton Dickinson, San Diego, CA).

For conjugation measurement, CTL clone OE4 (2.5 × 10^6) and A20.2J (2.5 × 10^6) in complete medium, in the presence or absence of 2 nM EDTA or EGTA, were mixed and spun at 1000 rpm for 1 min. After 30 min at room temperature, cells were resuspended and applied to flow cytometry. Data were analyzed with CellQuest software (Becton Dickinson), and the high light-scattering population was counted as conjugates.

DNA degradation assay
DNA degradation was measured by release of radioactivity from labeled target nuclei as previously described (21). Briefly, target cells were incubated in the presence of [153]IUdR (BM.355; Amersham, Little Chalfont, U.K.). Washed cells (1 × 10^5) were incubated with either anti-Fas mAb, Jo2, or Fas-dependent CTL P0K in 96-well plates. After 6 h incubation, cells were lysed by addition of Nonidet P-40 (0.1% final), and the released radioactivity in the supernatant was measured.

Assay for caspase-3-like activity
Fas-induced activation of caspase-3-like activity in A20.2J and its variant 1L1-A20-Jo2R was measured by cleavage of a tetra-peptide substrate according to the previous report (22). Cells (7 × 10^6) were treated with or without Jo2 mAb for 2 h and then lysed in 300 μl of lysis buffer (0.5% Nonidet P-40, 0.5 mM EDTA, 0.15 M NaCl, 50 mM Tris/Cl, pH 7.5). The 50-μl aliquot of lysate was mixed with 150 μl substrate solution (40 μM Ac-DEVD-MCA, 0.05 M NaCl, 2.5 mM DTT, 10 mM HEPES, pH 7.5) and incubated at 37°C for 2 h. Fluorescence of released 7-amino-4-methylcoumarin was measured by FluoroScan fluorescence microplate reader with 355 nm excitation and 460 nm emission filter set. The activity was expressed as the F.I. with arbitrary units.

Results
Plasma membrane labeling probe, FM1-43, is translocated into target cell intracellular membrane structures by CTL attack
The perforin-positive murine CTL clone OE4 (20) was incubated with an Ag-specific target A20.2J in media containing FM1-43, and changes in the membrane distribution of the dye was visualized by confocal microscopy. One minute after CTL-target contact, FM1-43 fluorescence became visible inside the target cell, and the total F.I. was dramatically increased by 3 min (Fig. 1). The images were obtained at room temperature, because kinetics of the process at 37°C was too fast for observation and analysis. We did

**FIGURE 1.** Translocation of cell-surface fluorescence labeling into intracellular membrane structures. Fluorescence of the cell surface of a B lymphoma A20.2J by FM1-43 translocated within 3 min upon being attacked by a CTL clone OE4. Time 0 was defined as the moment that CTL-target contact was observed. Images are shown in both confocal fluorescence (upper panels) and DIC micrograph (lower panels). This series of images is a representative of many similar observations. Bar, 10 μm.
not examined at 4°C, because it is known that mobility and function of CTL is completely abrogated at 4°C.

The dye appeared to form an overlapping pattern of punctate stains and associated with intracellular membrane structures such as the nuclear envelope (Fig. 1). Within this time frame, morphological changes typical to apoptosis were not observed by differential-interference-contrast (DIC) microscopy (Fig. 1). Because soluble FM1-43 present in the media is unable to diffuse across plasma membrane, the rapid translocation could be due either to influx of the free dye through pores generated by polyperforin, to the release of the dye from internalized vesicles damaged by perforin (9), or to fusion of internalized vesicles with intracellular structures and lateral diffusion of the dye.

FIGURE 2. Failure of detection of breaks on target cell surface by HPTS translocation in perforin-mediated cytotoxicity. A, Sublytic dose of purified human perforin induced translocation of FM1-43 but not of HPTS in Jurkat cell. Fluorescence images of FM1-43 (red) and HPTS (green) are superimposed. Thus, areas positive for both fluorescence are seen yellow. Images of just after addition of perforin (a) and 50 min after addition of perforin (500 U/ml) (b) are shown. Lysis under this condition was <10% in parallel assay. Subsequent permeabilization of cell membrane by digitonin (0.2 mM) caused massive stain by both HPTS and FM1-43 (c). Because the FM1-43 translocation was detectable within 10 min and the F. I. was increased linearly, the image just before the addition of digitonin (50 min) is shown as a representative. Cells with typical changes are indicated by arrows. B, Translocation of FM1-43 induced by FasL-defective perforin-dependent CTL clones. CTL clones GD1 and GD4 were incubated with either A20.2J or a FcR/Fas“ mast cell line PT18 in the presence or absence of anti-CD3 mAb 2C11 that provoke activation of T cells when immobilized on the cell surface via FcR. After collecting time-lapse confocal images, F.I. in the target cells including both plasma membrane and intracellular structures were calculated. Open symbols represent the absence of CTL or 2C11, F. I. of each time point was expressed as relative value to that of time 0. The number of cells analyzed for each series is shown in parenthesis. SD of each point was always 0.33. C, Breakage of the plasma membrane was not detected by influx of extracellular fluorescent dye HPTS. Two to 3 min (early phase) and about 15 min (mid phase) after contact between perforin-dependent CTL clone GD1 and A20.2J, images by DIC and confocal fluorescence microscopy (HPTS) were obtained at each focal depth indicated on the right. D, Influx of HPTS indicating membrane damage was observed only when nuclear deterioration (disappearance of fluorescence of ethidium, an enzymatic cleavage product of DHE, from the cells) occurred in the late stages of CTL-mediated killing. Target cell A20.2J was labeled with nuclear staining probe DHE, and unlabeled CTL clone OE4 was added at time 0.

The translocation of FM1-43 occurs in the absence of perforation or breakage of plasma membrane

To determine whether CTL-induced staining of intracellular structures with FM1-43 was due to influx of the dye through pores or breakage on plasma membrane, permeabilization of the target cell during perforin-dependent cytotoxicity was monitored using a membrane impermeant dye, HPTS (Fig. 2, A and C). Because HPTS is fluorescent in aqueous phase, only the extracellular compartment is fluorescent if the plasma membrane is intact (Fig. 2A, top panel). The application of a sublytic dose of purified perforin resulted in the FM1-43 translocation without influx of HPTS. While FM1-43 translocation was evident in almost all cells treated with 500 U/ml perforin (Fig. 2A, middle panel), <10% were lysed in a parallel assay (data not shown). However, the subsequent addition of digitonin induced a striking influx of both HPTS and FM1-43 (Fig. 2A, bottom panel). Thus, when the plasma membrane was damaged, HPTS indeed was able to diffuse to cytosol.

This observation was confirmed in cell-mediated cytotoxicity. CTL clones whose cytotoxicity is solely dependent on granule-based pathway (21, 23) were stimulated either with Ag-specific targets or with targets that were precoated with anti-CD3 mAb to bypass the specific Ag recognition. The specific cytolytic activity
of these clones has been shown to be solely dependent on the granule-based pathway (23). The FasL-defective CTL was able to induce translocation of FM1-43 in both target cell (Fig. 2B). The translocation measured by the increment of intracellular F.I. was detectable within minutes after CTL-target contact, as shown in Fig. 1, and it continued for at least 30 min (Fig. 2B). Permeabilization of the target cell was also examined with HPTS (Fig. 2C). At any focal plane analyzed immediately after CTL-target contact (2–3 min) or at 15 min, there was no detectable influx of HPTS into the target cytosol (Fig. 2C), whereas translocation of FM1-43 fluorescence was readily apparent (Fig. 2B). In the CTL-mediated cytotoxicity, HPTS was indeed capable of penetrating into target cells, but only in very late stages and it coincided with DNA damage shown by disappearance of DHE-labeled nuclei (Fig. 2D, 5 h). Therefore, these data suggest that perforin incorporation in the plasma membrane stimulates internalization of membrane-associated FM1-43 in the absence of detectable membrane permeabilization.

The absence of membrane permeabilization was also confirmed by showing that fluorescent-conjugated cytochrome c (4 kDa) and dextrans (20 kDa) failed to enter target cells, and 5-carboxyfluorescein (5-CF) or quinacrine remained within preloaded cells (Table I). The presence of damaged plasma membrane also was not detected when evaluated with several other fluorescent probes of intracellular and extracellular components (Table I). Therefore, it is confirmed that the translocation of FM1-43 precedes the damage of the cell membrane that breaks barrier function of the plasma membrane and is not attributable to the influx of the dye through pores.

Results with irreversible fluorescent membrane labeling probe, DiIC12 (18), suggest that CTL-mediated killing stimulates internalization of the membrane components of target cells (Table I and Fig. 3). A20.2J cells were labeled with DiIC12, washed, and then incubated with CTL clone OE4. Although DiIC12 stained both target cell plasma membrane and intracellular structures (Fig. 3A), an increase in intracellular fluorescence occurred within 4 min after adhesion of the CTL to the target cell and was associated with a reduction in cell-surface fluorescence (Fig. 3, A and B), whereas such translocation was not observed in cells not contacted with CTL (Fig. 3B). This result suggested that the target cell is stimulated to rapidly internalize its plasma membrane components following contact with CTL. In addition, the inward translocation of membrane-bound ConA (Table I) not only supports the translocation of membrane components but also indicates that membrane-associated macromolecules can also be translocated to internal structures. Taken together, these data suggest that CTL attack to the target cell and perforin incorporation in plasma membrane stimulate internalization of membrane-associated FM1-43 in the absence of detectable membrane permeabilization.

**Translocation of FM1-43 is also induced in Fas-dependent apoptosis**

To determine whether FM1-43 translocation was unique to the granule/perforin-mediated pathway or occurred also during Fas-dependent cell death, we studied targets bound to a perforin-deficient CTL line, POK (3). These effector cells induced an increase in F.I. in FcR+ A20.2J target cells coated with anti-FcD3 mAb 2C11 (Fig. 4A). A moderate F.I. increment was observed even in the absence of 2C11 (Fig. 4A). Because FasL- CTLs are known to kill Fas+ target cells nonspecifically, albeit with reduced efficiency (data not shown), the observed increment of FM1-43 fluorescence is attributable to the interaction of POK-associated FasL with Fas on the nonspecific target cell. To further establish that FM1-43 translocation occurs during Fas-mediated apoptosis, A20.2J cells were then treated with an anti-Fas mAb and changes in F.I. were evaluated. As shown in Fig. 4B, an increase in F.I. was demonstrated in the absence of the cytotoxic effector cells. These data indicate that the signal delivered by ligation of the Fas receptor also induces the translocation of FM1-43 and suggest that uptake of the dye may not be unique to granule-mediated apoptosis and not due to conjugation of the CTL and target cell. However, the kinetics of FM1-43 internalization appeared to be delayed compared with the changes observed during perforin-dependent cytotoxicity.

**Translocation of FM1-43 requires execution of CTL-mediated killing, and the mere conjugation of CTL and target is not sufficient**

Experiments were then undertaken to clarify if the translocation of the membrane-associated FM1-43 in the target cell occurred merely as a consequence of adhesion to the CTL or was a response to the cytotoxic process. This was accomplished by performing the study in the absence of extracellular free calcium ion, which is required for the execution of CTL killing but not CTL-target conjugate formation (Fig. 5A) (24). The depletion of extracellular free calcium by EGTA completely abrogated translocation of FM1-43 (Fig. 5B), while CTL-target conjugate formation was reduced by only 40% (Fig. 5A). In addition, the noncytotoxic Th2 clone
D10.G4 was also found not to induce translocation in the A20.2J cells prearmed with anti-CD3 mAb (Table II), further supporting that FM1-43 translocation accompanied delivery of the lethal hit. Therefore, these results suggest that induction of the FM1-43 translocation is not a simple response to adhesion of the CTL to the target cell, rather is associated with delivery of the "lethal hit." FM1-43 translocate to Mt

Mt permeability transition is reportedly a central event during many forms of apoptosis (25). Because the translocated FM1-43 appeared to associate with Mt, the localization of translocated FM1-43 was monitored in target cells labeled with MitoTracker dyes that are Mt-specific fluorochromes that remain confined to Mt after washing. Within 10 min, FM1-43 appeared to coalesce with Mt (yellow-orange spots in Fig. 6) in target cells that had been attacked by CTL, but not in target cells not attacked by CTL (not shown), indicating that FM1-43 was incorporated into Mt during an early stage of CTL-induced apoptosis. Although some spots dissappeared or appeared in the course of observation, it is likely due to the movement of Mt across the focal plane, because the cells were vitally stained and it is known that Mt is mobile in live cells. Such movement may also be caused by distortion of the target cell due to CTL attack.

**FM1-43 translocation occurred in the absence of caspase-3-like activity**

We have isolated a variant of A20.2J, 1L1-A20-Jo2R, that expresses Fas and FcR at the same level as the parental A20.2J (Fig. 7 A) but was not sensitive to Fas-induced apoptosis in both anti-CD3 mAb 2C11 or Jo2. F.I. of each time point was expressed as relative value to F.I. of time 0. The number of cells analyzed for each series is shown in parenthesis. SD of each point was always <0.31.

**FIGURE 3.** Translocation of irreversible fluorescent membrane labeling dye DiIC12. A, A20.2J labeled with DiIC12 was washed to remove the excess of the dye before adding CTL clone OE4. F.I. of confocal images of a target/CTL conjugate were visualized by pseudo-colored representation. Bar, 10 μm. B, Changes of F.I. in the plasma membrane, in nuclear envelope, and of the whole target cell. F.I. was calculated with whole cell area of digitized confocal image, as well as with area of 0.5 × 0.5 μm or of 0.75 × 0.75 μm in the plasma membrane and in nuclear envelop, as indicated by closed arrowhead and open arrowhead in A, respectively. The F.I. at time 0 of each area was defined as 1, and the relative F.I. are plotted. Average values of three targets that were attacked by CTL (solid lines) and those of eight targets that were not contacted with CTL (dashed gray lines) were plotted. Error bars represent SEM. Cells not contacted with CTL did not show any changes in DiIC12 fluorescence.

**FIGURE 4.** Translocation of FM1-43 induced by Fas ligation. Changes of F.I. in A20.2J induced by a perforin-deficient FasL-dependent CTL line P0K (A) and an anti-Fas mAb Jo2 (B) were measured and plotted as Fig. 2B. Open symbols represent the absence of anti-CD3 mAb 2C11 or Jo2. F.I. of each time point was expressed as relative value to F.I. of time 0. The number of cells analyzed for each series is shown in parenthesis.

**FIGURE 5.** CTL-target conjugation and the translocation of FM1-43. A, Conjugate formation was measured by flow cytometry in the absence or presence of 2 mM EDTA or EGTA. Events in the polygon frames in forward- and side-scatter plots were regarded as CTL-target conjugates, and the percentages are indicated. B, Aliquots of samples for A were in parallel examined under confocal fluorescence microscopy 30 min after addition of FM1-43, and the percentage of cells in which FM1-43 translocation was observed were plotted. Because minute translocation was observed in ~10% of A20.2J that did not contact with CTL, such incidence was not counted in each data. The percentage of conjugate in the suspension obtained from A is also plotted for comparison.
Fas-induced (Fig. 7B) and Fas-dependent CTL-mediated systems (Fig. 7C). Activation of a family of proteases, caspases, is also considered indicative of an apoptotic response. However, this variant failed to activate caspase-3 activity upon Fas-mediated signaling (Fig. 7D), suggesting defects in the apoptosis signaling pathway upstream of caspase-3 activation. However, Fas-dependent CTL-induced FM1-43 translocation was equally detectable in both A20.2J and 1L1-A20-Jo2R variant (Fig. 7E), indicating that the translocation of FM1-43 is a very early event and independent of the caspase-3-like activity.

Discussion

We show here for the first time that during CTL attack the target cell undergoes pronounced alteration in intracellular membrane trafficking. FM1-43 has been used extensively to study and dissociate the exocytic and endocytic events in numerous secretory cells (12–15). The translocation of the dye may occur through association with the plasma membrane remnant of the endocytic vesicle, through influx of soluble dye past transmembrane pores, and through uptake of soluble dye by fluid-phase endocytosis. In most reports, internalization of FM1-43 is associated with the development of a pattern of punctate stains consistent with a vesicular distribution (26). In contrast, in the images generated after CTL contact, there was an unanticipated dramatic increase in staining of the nuclear membrane and mitochondria in the absence of detectable evidence for perforation or breakage of plasma membrane.

Five possibilities might account for this massive transfer of the dye. First, the dye may be released from perforin-damaged vesicles and diffuse to intracellular structures. Second, the fusogenic potential of the endocytic vesicles may be enhanced by incorporation of proteins delivered by the CTL facilitating fusion with intracellular organella. Subsequently, through lateral diffusion, FM1-43 associated with the membranous components of the organella. Third, massive fusion among membranous components of the cell

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** FM1-43 translocation into Mt. Target cell A20.2J prestained with MitoTracker Red was mixed with CTL clone OE4 in the presence of FM1-43. Fluorescence of FM1-43 and Mt-specific dye MitoTracker is indicated as green and red, respectively, and the both images are superimposed. Therefore, colocalization of both dyes is seen as yellow-orange. The moment when an OE4 (indicated as CTL1) contacted with an A20.2J cell was defined as time 0. DIC images (lower panels) and confocal fluorescence images (upper panels) are shown with the resolution of 0.041 μm/pixel.

**FIGURE 7.** Caspase-3-independent induction of FM1-43 translocation observed with a Fas-resistant variant cell line. A, Expression levels of Fas and FcR on A20.2J and an its variant 1L1-A20-Jo2R were not significantly different. Broken lines, specific mAb against Fas or FcR; solid lines, negative control mAb. The variant 1L1-A20-Jo2R was resistant to Fas-mediated apoptosis induced by anti-Fas mAb, Jo2 (B), and by Fas-dependent CTL line P0K (C). P0K-mediated killing was performed in the presence of 2C11 to bypass the Ag recognition. D, Treatment with anti-Fas mAb caused strong activation of caspase-3-like activity in A20.2J, whereas the activity was totally absent in the variant 1L1-A20-Jo2R. E, Translocation of FM1-43 induced by Fas-dependent CTL P0K was equally detected in both A20.2J and 1L1-A20-Jo2R in the presence of 2C11.
including plasma membrane is possible. The translocation of DiIC12 (Fig. 3) and cell-surface glycoconjugates labeled with FITC-suc.ConA into the intracellular structures (data not shown, Table I) was observed. These findings are consistent with a membrane fusion model. If this is truly the case, the inward translocation through the membrane connection might enable cell membrane components, e.g., proteins and lipids, to migrate directly to membranes of intracellular organella, bypassing cytosolic signal cascades. Fourth, membrane perturbation such as expression of PS on the cell surface (10, 11) and activation of sphingomyelinase (27–29) may contribute to cell death-associated translocation of fluorescent labels described in this report. Because FM1-43 is incorporated into the outer layer of plasma membrane (13), the dye might be able to diffuse to intracellular organella only if the molecule “flips” to the inner layer of plasma membrane (10, 11). The “flip-flop” of membrane components facilitated by certain “flipase” or “scramblase” activity (30, 31) might lead to the internalization of FM1-43-associated lipids. Such a mechanism may also contribute to the exposure of PS on the cell surface, and indeed we observed in preliminary analysis that kinetics of annexin V binding was similar with that of the FM1-43 translocation. However, because annexin V binding is reported to be dependent on caspase activation (32), the exposure of PS is likely to be independent of FM1-43 translocation. Finally, FM1-43 may enter cells through a certain type of cation channel, as reported for a divalent cation channel operated in mechanosensitive cell of Xenopus larvae (33). Although influx of extracellular calcium in the target cell was reported to be detectable in CTL-mediated killing (34), it was not detected in our analysis with perforin-positive CTL clones (Table I). Thus, the influx of FM1-43 through the calcium channel is unlikely under this condition.

On the basis of recent studies on the granule-dependent pathway of CTL-mediated killing (5, 35, 36), we have postulated that perforin facilitates the intracellular delivery of the granzymes through an endosomolytic mechanisms (9). To our knowledge, this is the first report to show that the target does not undergo extensive permeabilization during perforin-dependent CTL attack (Fig. 2, C and D). Therefore, entry of FM1-43 through transmembrane pores is unlikely. In addition, the finding that isolated perforin also fails to permeabilize the target cells (Fig. 2A) is consistent with a recent report where sublytic amounts of perforin was sufficient to deliver granzyme B and induce apoptosis without the genesis of pores allowing influx of fluorescent markers (8). In the absence of obvious membrane permeabilization, it appears that FM1-43 enters the target through either fluid-phase endocytosis or in membrane-associated form during receptor-dependent endocytosis with constituents released by the granules of the CTL. However, to explain the rapid redistribution of the dye would require the intracellular release of FM1-43 and diffusion of the dye to Mt and nuclear membranes. This possibility is consistent with the postulated endosomolytic effect of perforin and the observation that vesicle-associated granzyme B is rapidly released to the cytosol and translocated to the nucleus following the application of perforin to target cells (6, 8). Nevertheless, because internalized DiIC12 also undergoes intracellular redistribution during CTL-mediated apoptosis (Fig. 3), the results agree with the secondary mechanism that depends on extensive fusion of internalized vesicles with intracellular organella. Such fusion is also suggested by a model system in which apoptosis of COS cells transiently expressing only intracellular granzyme B was induced by the addition of perforin (6). Thus, the results presented here suggest two distinct mechanisms on the delivery of proapoptotic granule constituents at an intracellular level: internalized perforin releases the coincidentally endo-
understand the intracellular trafficking events during this crucial biologic process.

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