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Structural Change of Ribosomes during Apoptosis: Degradation and Externalization of Ribosomal Proteins in Doxorubicin-Treated Jurkat Cells[1]

Jun Nishida,* Akiko Shiratsuchi,† Daita Nadano,‡,2 Taka-aki Sato,§ and Yoshinobu Nakanishi†,3

**Graduate School of Natural Science and Technology and †Graduate School of Medical Science, Kanazawa University, Kanazawa, Ishikawa 920-0934; ‡Molecular Oncology Laboratory, Tsukuba Institute, RIKEN, Tsukuba, Ibaraki 305-0074; and §Department of Otolaryngology/Pathology, Columbia University, New York, New York 10032, USA*

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Changes in the amount and localization of human ribosomal proteins during apoptosis were determined. When total lysates of Jurkat cells undergoing apoptosis induced by doxorubicin were analyzed by Western blotting, degradation of three ribosomal proteins, S18, L5, and L14, was detected at 48 h after the induction of apoptosis. Decreases in the amounts of these three ribosomal proteins were also observed in ribosome-enriched fractions. These changes were partly abolished by the addition of the pan-caspase inhibitor z-VAD-fmk. Moreover, formation of the 80S ribosome complex appeared to be inhibited at 48 h after apoptosis induction. On the other hand, the rate of protein synthesis, assessed by measuring the incorporation of [^{35}S]Met into bulk proteins, decreased as early as 12 h after the addition of doxorubicin. These results indicate that changes in the amount of ribosomal proteins and the overall structure of ribosomes in apoptosing cells occur after protein synthesis declines. Finally, analyses by flow cytometry, immunofluorescence, and Western blotting showed that six ribosomal proteins, S15, P0, L5, L6, L36a, and L41, were relocalized and expressed at the cell surface during apoptosis. The above results collectively indicate that ribosomes are structurally altered in apoptotic cells following inactivation of protein synthesis.

Key words: apoptosis, protein degradation, protein synthesis, ribosomal protein, ribosome.

Degradation of chromosomal DNA at the last stage in the process of apoptosis should result in the complete cessation of gene expression in cells fated to die. However, the rate of protein synthesis decreases even before chromosomal DNA is degraded (*1–4*), suggesting the presence of an active mechanism that inhibits gene expression at the level of protein synthesis in apoptotic cells. The molecular basis for this event, however, is not fully understood.

A variety of proteins and nucleic acids are involved in protein synthesis. The ribosome stands at the center of the translational machinery, and the eukaryotic 80S ribosome complex consists of two subunits, 40S and 60S, each of which contains a large number of proteins and RNA. Although the precise function of individual ribosomal proteins and RNA has not yet been clarified, it is reasonable to expect that the inhibition of protein synthesis in apoptotic cells is the consequence of structural modification of these molecules. In fact, the 28S ribosomal RNA is selectively degraded in apoptotic cells (*3, 5–7*). This event occurs at relatively early stages of apoptosis *via* both caspase-dependent and -independent mechanisms. It is, however, not clear whether degradation of the 28S RNA leads to structural changes of ribosomes or the inhibition of protein synthesis. On the other hand, factors that are closely related to the translation reaction have been shown to undergo degradation in apoptotic cells: eukaryotic translation initiation factors eIF2, eIF3, eIF4B, and eIF4G are degraded in a manner dependent on caspases (*2, 4, 8, 9*). The loss of such factors explains, at least in part, the decrease in the rate of protein synthesis during apoptosis.

In contrast, changes in ribosomal proteins appear to be more complex. There are two reported examples of ribosomal proteins that are released from the ribosome and relocated in apoptotic cells. Rosen and colleagues reported that S1, a ribosomal protein present in the 40S ribosome subunit, became detectable in the membrane bleb at the surface of apoptotic cells, suggesting that ribosomal proteins are relocated during apoptosis (*10*). The second example of a ribosomal protein that changes its location during apopto-

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[2] Present address: Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601.

[3] To whom correspondence should be addressed. Tel: +81-76-234-4481, Fax: +81-76-234-4480, E-mail: nakanaka@kenroku.kanazawa-u.ac.jp

Abbreviations: FITC, fluorescein isothiocyanate; PS, phosphatidylserine; SDS, sodium dodecyl sulfate.

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sis is S19; it appears to be covalently dimerized and secreted from apoptotic cells (*11–14*). Moreover, the modified S19 serves as a chemoattractant specific for macrophages (*11*). Two more reports suggest the structural modification of ribosomes during apoptosis: cleavage or at least modification of the 60S ribosome subunit protein P0 (*15*) and destruction of polysomes (*1*). These findings indicate that ribosomal proteins and the ribosome complex are structurally modified in apoptotic cells, and suggest that some ribosomal proteins have extra-ribosomal functions. However, the previous studies dealt with only a limited number of ribosomal proteins, and changes in the overall structure of the ribosome in apoptotic cells have not been intensively investigated. Nadano *et al.* recently examined structural changes of ribosome subunits and proteins using anti-ribosomal protein antibodies and found that the amount of S11 decreases in human tumor–derived cells undergoing staurosporine-induced apoptosis (*16*). This change seemed to occur in a cell type–specific manner: it was evident in four different cell lines derived from human breast carcinoma, but not in cells from other types of tumors (*16*). Here we analyzed the amount, structure and localization of ribosomal proteins in apoptotic human leukemia cells using polyclonal antibodies raised against peptides corresponding to partial amino acid sequences of 79 human ribosomal proteins.

MATERIALS AND METHODS

Cell Culture and Induction of Apoptosis—Human T-cell leukemia Jurkat cells were maintained in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo) with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air. The cells (2×10^5 cells/ml) were induced to undergo apoptosis by treatment with the DNA-intercalating anticancer drug doxorubicin (*17*) (0.3 μg/ml) (Sigma, St. Louis, MO, USA) or the agonistic anti-human Fas monoclonal antibody CH11 (*18*) (0.1 μg/ml) (MBL, Nagoya). When ribosome-enriched fractions were prepared (see below), Jurkat cells at a density of 1×10^6 cells/ml were treated with 1.5 μg/ml doxorubicin. The progress of apoptosis was almost the same under these conditions. The induction of cell death was assessed by measuring the exclusion of trypan blue from cells (integrity of the plasma membrane), the stainability of the nucleus with Hoechst 33342 (condensation of chromatin), and the binding of fluorescein isothiocyanate (FITC)–labeled annexin V to the cell surface [externalization of the membrane phospholipid phosphatidylserine (PS)] as described previously (*19*). To inhibit caspase-dependent apoptosis, Jurkat cells were pre-incubated for 1 h with the pan-caspase inhibitor z-VAD-fmk (20 μM) (Peptide Institute, Osaka), then treated with doxorubicin in the presence of the inhibitor.

Preparation of Protein Samples for Analysis of Ribosomal Proteins and Subunits—Whole-cell lysates were obtained by lysing cells directly with the buffer for sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis [62.5 mM Tris-HCl (pH 6.8), 2.5% SDS, 2.5% 2-mercaptoethanol]. The fractions enriched in ribosomes were prepared from the liver of adult male Donryu rats or Jurkat cells according to the method of Bommer *et al.* (*20*). Briefly, tissues or cells were homogenized in a buffer containing 0.25 M sucrose and centrifuged at 12,000 ×*g*. The post-mitochondrial supernatants were layered on a buffer containing 1 M sucrose and centrifuged at 260,000 ×*g*. The pellets were suspended in a buffer containing 0.25 M sucrose and sedimented again through 1 M sucrose. The final pellets were collected and used as the ribosome fraction. To obtain cell surface proteins, Jurkat cells that had been treated with doxorubicin (0.3 μg/ml) for 24 h and still possessed intact plasma membranes were labeled with biotin (NHS-LC-Biotin; Pierce, Rockford, IL, USA), lysed in a buffer consisting of 10 mM Tris-HCl (pH 7.8), 1% Nonidet P-40, 0.25 M NaCl, and 1 mM EDTA, and centrifuged. The supernatants were mixed with streptavidin-conjugated magnetic beads (Dynabeads M-280 Streptavidin; Dynal, Oslo, Norway), and bound proteins were collected and used as the cell surface proteins.

Western Blotting—Anti–human ribosomal protein antibodies were obtained by immunizing rabbits with keyhole limpet hemocyanin-conjugated synthetic peptides that contained amino acid sequences (10–13 residues) corresponding to 33 small subunit and 46 large subunit proteins of the human ribosome, as described previously (*21*). Protein samples were electrophoresed on a 15% SDS–polyacrylamide gel, and the separated proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA, USA). The membrane was first reacted with an anti–ribosomal protein antiserum, then with alkaline phosphatase–conjugated anti–rabbit IgG (Bio-Rad, Hercules, CA, USA), and signals were chemiluminescently visualized using Immun-Star substrates (Bio-Rad). In some experiments, the specificity of the antibody binding was examined by pre-incubating the first antibodies for 1 h with excess amounts of the corresponding antigen peptides prior to the reaction with the membrane. To analyze the formation of the ribosome complex, we employed a newly developed method (*16*). Ribosome-enriched fractions were electrophoretically separated on a composite gel consisting of polyacrylamide (2%) and agarose (0.7%), and ribosome subunits were detected by Western blotting as described above.

Analysis of Protein Synthesis—Jurkat cell cultures were exposed to doxorubicin (0.3 μg/ml) for various periods, then pulse-labeled for 20 min in Met-free RPMI 1640 medium (Sigma) containing L-[^{35}S]Met (110 μCi/ml) (EasyTag; NEN Life Science Products, Boston, MA, USA). The harvested cells were lysed by the addition of 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100 and centrifuged, the supernatants were separated by electrophoresis in a 10% SDS–polyacrylamide gel, and the proteins were visualized by autoradiography. Cell-free protein synthesis was carried out using a reticulocyte lysate system (Promega, Madison, WI, USA). The lysates were incubated with oligo d(T)-selected Jurkat cell RNA (0.2 mg/ml) and a mixture of amino acids including L-[^{35}S]Met in the presence or absence of doxorubicin (0.3 μg/ml) at 30°C for 90 min, and the products were detected as described above.

Flow Cytometry—Jurkat cells were harvested, washed with phosphate-buffered saline, and successively reacted with anti–ribosomal protein antisera and FITC-labeled anti–rabbit IgG (Immunotech, Marseilles, France). The cells were then analyzed in a flow cytometer (Epics-XL; Coulter, Hialeah, FL, USA). To examine the specificity of the binding of the first antibodies, the reaction was done in the presence of the corresponding antigen peptides.

Immunofluorescence—Cells were smeared on 3-aminopropyltriethoxysilane–coated slide glasses and successively treated with anti–ribosomal protein IgG that had been prepared by protein A–Sepharose chromatography, a biotinylated anti–rabbit IgG antibody (Vector, Burlingame, CA, USA), and Alexa Fluor 488–conjugated streptavidin (Molecular Probes, Eugene, OR, USA). The samples were washed and examined under a confocal laser scanning microscope (LSM 510; Carl Zeiss, Jena, Germany). To simultaneously detect a ribosomal protein and a cell surface protein, cells were treated with the anti-L5 antibody and the anti-Fas antibody CH11, and smeared on slide glasses. They were further reacted with a biotinylated anti–rabbit IgG antibody/Alexa Fluor 488–conjugated streptavidin and Cy3-conjugated anti–mouse IgM (Jackson ImmunoResearch, West Grove, PA, USA), and analyzed by confocal microscopy.

RESULTS

Detection of Ribosomal Proteins by Western Blotting—Antisera were obtained from rabbits that had been immunized with synthetic peptides corresponding to partial amino acid sequences of 33 small and 46 large human ribosomal subunit proteins, and tested for their ability to bind to native ribosomal proteins. For this purpose, whole-cell lysates of Jurkat cells and ribosome-enriched fractions of rat liver were analyzed by Western blotting. As shown in Fig. 1, both samples gave signals, though with varying intensities, at the positions corresponding to the expected molecular masses of 11 small subunit proteins, Sa, S3a, S4X, S8, S11, S12, S14, S15, S18, S24, and S30, and 17 large subunit proteins, L5, L7, L7a, L8, L9, L13, L13a, L14, L15, L17, L18, L28, L31, L34, L35a, L36a, and L39. The antisera that failed to react with the corresponding ribosomal proteins probably recognized only the structure specific to the antigen peptides. We then used the antisera recognizing the above 28 ribosomal proteins to determine whether the amounts of these proteins changed during the process of apoptosis.

Decrease in Amount of Ribosomal Proteins during Apoptosis—Jurkat cells were induced to undergo apoptosis by treatment with doxorubicin. Such treatment caused typical apoptotic changes in Jurkat cells, such as loss of plasma membrane integrity, chromatin condensation, and externalization of PS to the cell surface (Fig. 2). Whole-cell lysates were prepared from Jurkat cells that had been exposed to the drug for various lengths of time, and subjected to Western blotting with the 28 kinds of anti–ribosomal protein antisera (Fig. 3A). The antisera were reacted with the blotted membranes in the presence or the absence of the corresponding antigen peptides to test the specificity of the signals. Most ribosomal proteins did not show changes in response to doxorubicin, as exemplified by the result with the anti-S4X antiserum. However, the levels of S18, L5, and L14 were affected by doxorubicin: the amounts of S18 decreased, and signals with faster mobility appeared on the gels stained with anti-L5 and -L14 antibodies as the time of drug treatment was increased. These changes all became apparent at 48 h or later after the induction of apoptosis. It is thus likely that these three ribosomal proteins undergo degradation during apoptosis. We next examined whether

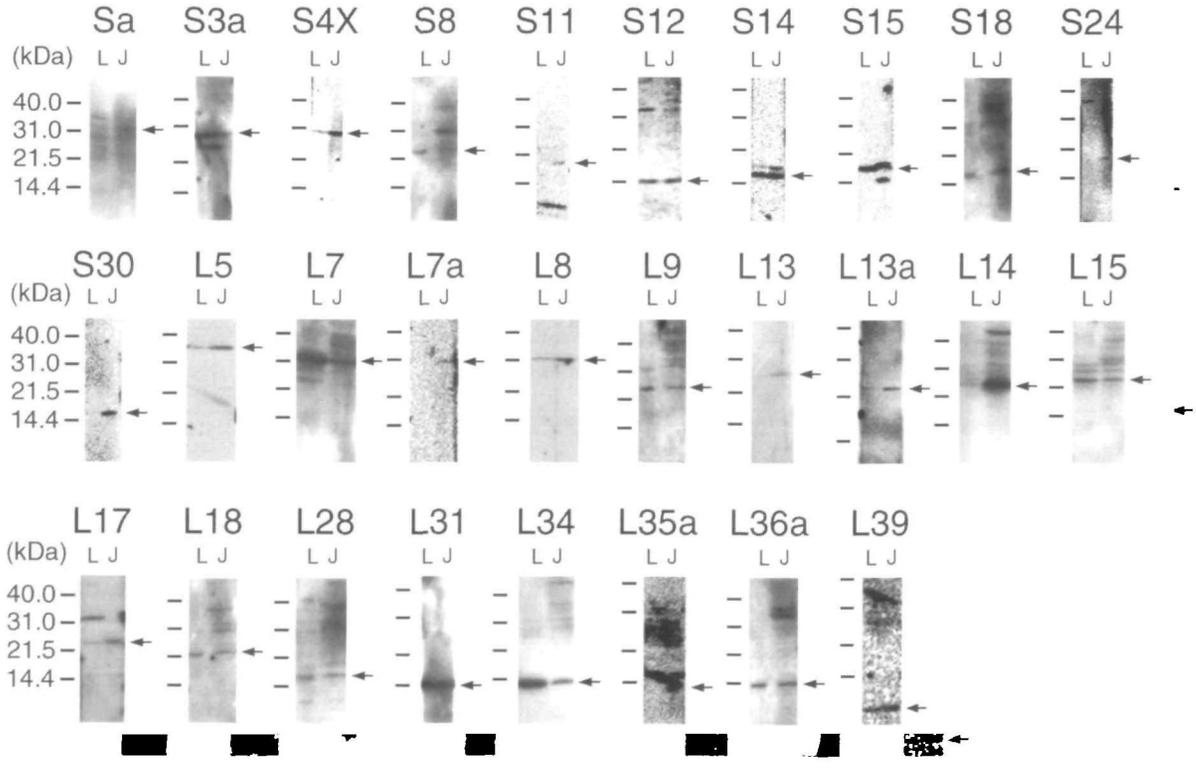

Fig. 1. **Detection of ribosomal proteins by Western blotting.** Ribosome-enriched fractions of rat liver (L) (10 or 75 μg protein) and whole-cell lysates of Jurkat cells (J) (40 or 80 μg protein) were analyzed with 79 kinds of anti-human ribosomal protein antisera. The results for antisera that gave specific signals are presented. The arrows indicate the positions of ribosomal proteins. The positions of molecular mass markers are shown at the left (in kilodalton).

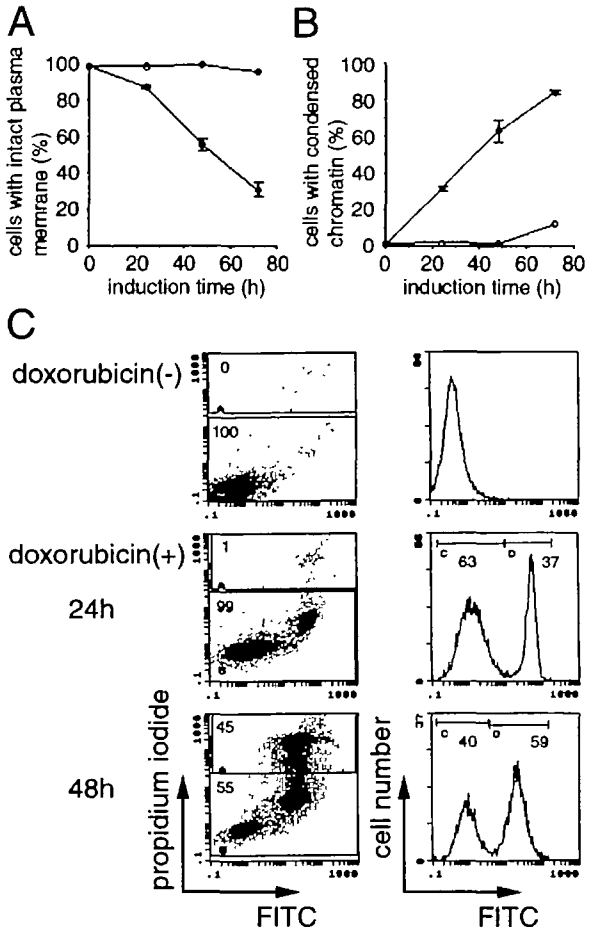

Fig. 2. **Induction of apoptosis in Jurkat cells by doxorubicin.** Jurkat cells were incubated with doxorubicin for the indicated periods and examined for biochemical changes indicating apoptosis. A: The integrity of the plasma membrane was examined by staining cells with the trypan blue dye. The relative number of trypan blue–negative cells is shown as a percentage. Closed and open circles indicate cells treated and not treated with doxorubicin, respectively. B: The occurrence of chromatin condensation was examined by staining cells with Hoechst 33342. The relative number of cells containing condensed chromatin is shown as a percentage. Closed and open circles indicate cells treated and not treated with doxorubicin, respectively. C: The presence of PS on the cell surface was examined in a flow cytometer by staining cells with FITC-labeled annexin V and propidium iodide. The numbers indicate the relative cell number (as a percentage) present in the corresponding areas. The propidium iodide–negative cells (bottom area in the left panels) were gated and re-plotted in the right panels, and the cells corresponding to the right peak possess externalized PS.

the same changes occurred in the ribosome complex. To do this, fractions enriched in ribosomes were prepared from apoptotic Jurkat cells by density gradient centrifugation and analyzed by Western blotting (Fig. 3B). The decreases in the amounts of the three proteins were observed with the ribosome fraction more significantly than with whole-cell lysates. However, the presumed degradation products of L5 and L14 were not detectable. These results indicate that three ribosomal proteins, S18, L5, and L14, are degraded upon induction of apoptosis, and suggest that the degraded proteins are dissociated from the ribosome complex, resulting in the appearance of ribosomes deficient in these proteins in apoptotic cells.

To examine whether these changes are restricted to cells undergoing doxorubicin-induced apoptosis, Jurkat cells were treated with the agonistic anti–human Fas antibody CH11, and their lysates were analyzed by Western blotting. As clearly seen in Fig. 4, almost the same changes were observed with cells undergoing Fas-mediated apoptosis. This indicates that degradation of the three ribosomal proteins occurs in apoptotic Jurkat cells independently of the mode of apoptosis induction.

We then examined whether such changes occurred during caspase-dependent apoptosis. Jurkat cells were treated with doxorubicin in the presence of the pan-caspase inhibitor z-VAD-fmk, and whole-cell lysates were analyzed for ribosomal proteins by Western blotting. Treatment with the caspase inhibitor reduced the fraction of cells with chromatin condensation to less than 20% (Fig. 5A). Under these conditions, the changes in the three ribosomal proteins were largely, though not completely, abolished (Fig. 5B). This indicates that the degradation of ribosomal proteins is partly dependent on the action of caspases.

Decrease in Amount of 80S Ribosome Complex in Apoptotic Cells—The loss of even a few kinds of ribosomal proteins might influence the structural integrity of the ribosome complex. To examine this possibility in apoptotic cells, we examined the presence of ribosome complexes in doxorubicin-treated cells. Ribosome-enriched fractions prepared from normal Jurkat cells were separated in an agarose/polyacrylamide composite gel and analyzed by Western blotting with anti–ribosomal protein antisera (Fig. 6A). Two distinct signals, which almost completely disappeared when the antibody reaction was carried out in the presence of the corresponding antigen peptides, were obtained with either the anti-S4X or anti-L34 antiserum. The signal with slower mobility was detected at almost the same positions with the two antisera and thus considered to be the 80S complex, which derived from either polysomes or the 80S ribosome pool. We concluded that the three signals were, in the order of their migration in the gel, derived from 40S, 60S, and 80S ribosome complexes.

We then tested the ribosome-enriched fractions prepared from Jurkat cells that had been exposed to doxorubicin for various lengths of time (Fig. 6B). When the samples were analyzed with the anti-S4X antiserum, the signal from the 80S complex decreased as the culture period was increased, whereas that from the 40S subunit was unchanged. Similarly, the analysis with the anti-L34 antiserum showed a decrease in the intensity of the 80S complex with a marginal change of the 60S signal. These changes became apparent at 48 h after induction of apoptosis, when ribosomal proteins S18, L5, and L14 started to be degraded. These results suggest that formation of the 80S complex was inhibited in the later stages of apoptosis.

Decline of Protein Synthesis at Early Stage of Apoptosis—To study the correlation between the above findings and changes in the rate of protein synthesis, Jurkat cells that had been treated with doxorubicin for various lengths of time were pulse-labeled with a radiolabeled amino acid, and the amount of labeled proteins was determined by SDS–polyacrylamide gel electrophoresis and autoradiography. As shown in Fig. 7A, signals derived from labeled proteins became weakened as early as 12 h, and undetectable at 24 h, after the induction of apoptosis. This appeared not

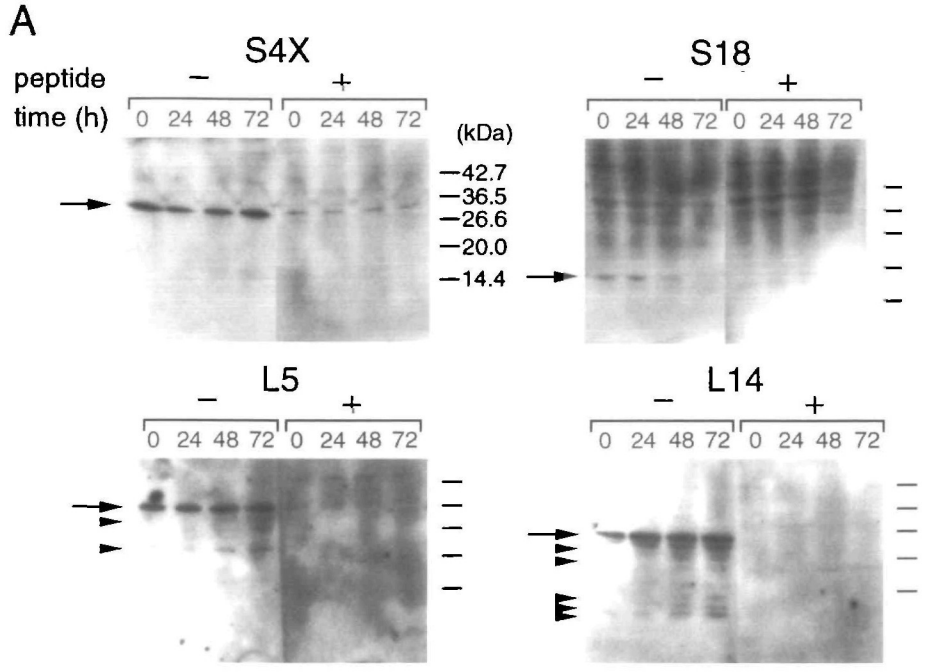

Fig. 3. **Changes in the amounts of ribosomal proteins in doxorubicin-treated Jurkat cells.** A: Whole-cell lysates of Jurkat cells that had been treated with doxorubicin for the indicated periods were analyzed by Western blotting with 28 kinds of anti-ribosomal protein antisera. Results showing changes in the signals of ribosomal proteins (S18, L5, and L14) together with an example of the results with no change (S4X) are presented. Reactions of the antisera with the blotted membranes were done in the presence (+) or absence (–) of the corresponding antigen peptides. The arrows and arrowheads indicate the intact ribosomal proteins and presumed degradation products, respectively. The positions of molecular mass markers are shown at the right of each panel. B: Ribosome-enriched fractions prepared from Jurkat cells that had been treated with doxorubicin were analyzed by Western blotting with the three antisera. The symbols are the same as those used in A.

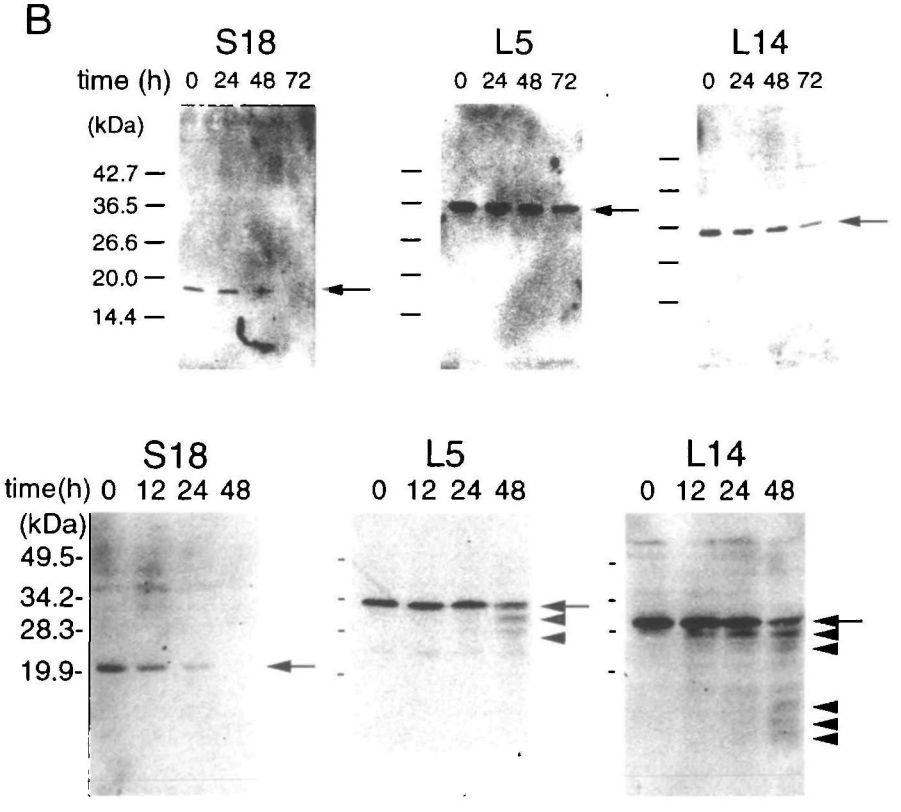

Fig. 4. **Changes in the amounts of ribosomal proteins in CH11-treated Jurkat cells.** Whole-cell lysates of Jurkat cells that had been treated with the anti-Fas antibody CH11 for the indicated periods were analyzed by Western blotting with three anti-ribosomal protein antisera. The symbols are the same as those used in Fig. 3A.

to be the direct effect of doxorubicin on translation, since protein synthesis in a cell-free system was not altered in the presence of the drug (Fig. 7B). These results indicate that the inhibition of protein synthesis during apoptosis precedes the structural changes of ribosomes, that is, the degradation of ribosomal proteins and the disappearance of the 80S ribosome complex.

Cell Surface Expression of Ribosomal Proteins—Some ribosomal proteins have been shown to be released from ribosomes during apoptosis. In particular, the small subunit protein S1 is transferred to the membrane bleb of apoptotic cells (*10*). We therefore examined whether any ribosomal proteins are expressed on the surface of apoptotic cells. Jurkat cells were treated with doxorubicin for 24 h, at which time they showed chromatin condensation and PS externalization but were not stained with propidium iodide (see Fig. 2), and were thus considered to have intact plasma membranes. These cells as well as normal Jurkat cells were

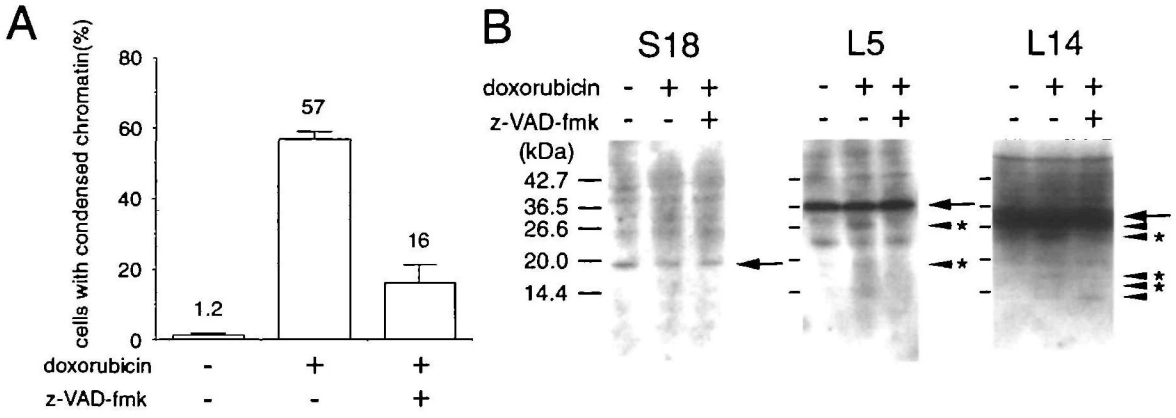

Fig. 5. **Caspase-dependency of change of ribosomal proteins in doxorubicin-treated Jurkat cells.** A: Jurkat cells were treated with doxorubicin for 48 h in the presence or absence of the pan-caspase inhibitor z-VAD-fmk. The fraction of cells containing condensed chromatin is shown. B: Whole-cell lysates of Jurkat cells that had been treated with doxorubicin for 48 h (L5 and L14) or 72 h (S18) in the presence or absence of the inhibitor were analyzed for changes of ribosomal proteins by Western blotting. The symbols are the same as those used in Fig. 3A except that the signals reduced by the inhibitor are asterisked.

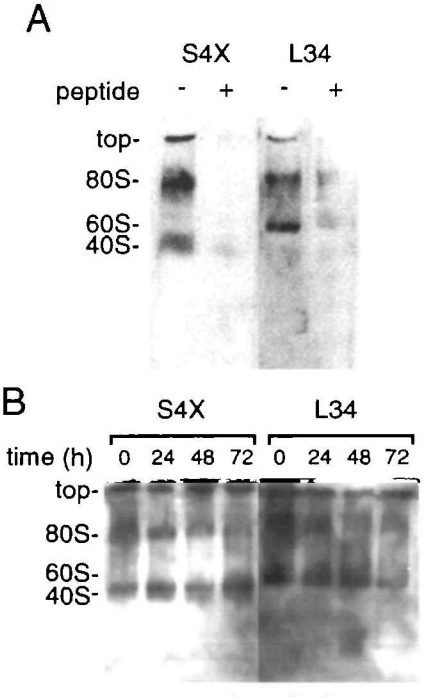

Fig. 6. **Failure of formation of the 80S ribosome complex in apoptotic Jurkat cells.** A: Ribosome-enriched fractions from normal Jurkat cells were separated on a composite gel consisting of agarose and polyacrylamide, and subjected to Western blotting with anti-ribosomal protein antisera. Incubations of the antisera with the blotted membranes were performed in the presence or absence of the corresponding antigen peptides. The positions of ribosome complexes and the top of gels are indicated at the left. B: Jurkat cells that had been treated with doxorubicin for the indicated periods were analyzed as in A.

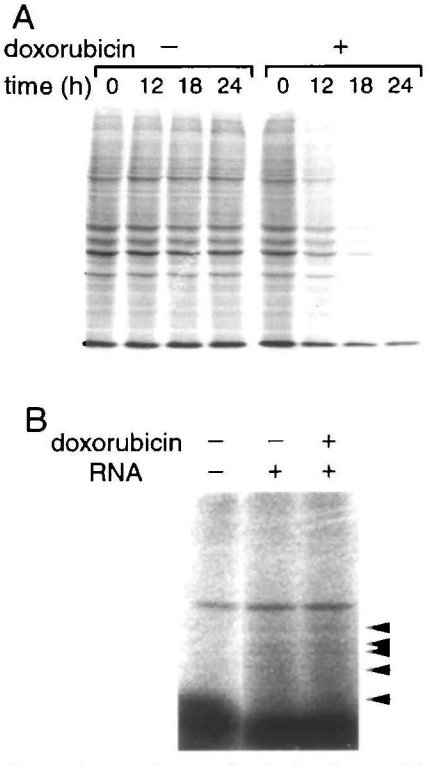

Fig. 7. **Decline of protein synthesis in doxorubicin-treated cells.** A: Jurkat cells treated with doxorubicin or left untreated for the indicated periods were analyzed for the rate of protein synthesis as described in the "MATERIALS AND METHODS" section. B: Cell-free protein synthesis with reticulocyte lysates was carried out in the presence or absence of doxorubicin and exogenously added RNA, and analyzed as in A. The arrowheads indicate RNA-dependent signals.

subjected to flow cytometry, immunofluorescence, and Western blotting with specific antibodies to determine the surface expression of ribosomal proteins.

We first determined which antisera were usable in flow cytometry. For this purpose, normal Jurkat cells were permeabilized by treatment with methanol and analyzed by flow cytometry with 79 kinds of antisera in the presence or absence of the corresponding antigen peptides. Twenty-eight antisera recognizing 10 small subunit proteins, Sa, S2, S4X, S11, S14, S15, S18, S24, S29, and S30, and 18 large subunit proteins, P0, P2, L3, L4, L5, L6, L7, L10, L14, L23a, L29, L35a, L36, L36a, L37, L38, L39, and L41,

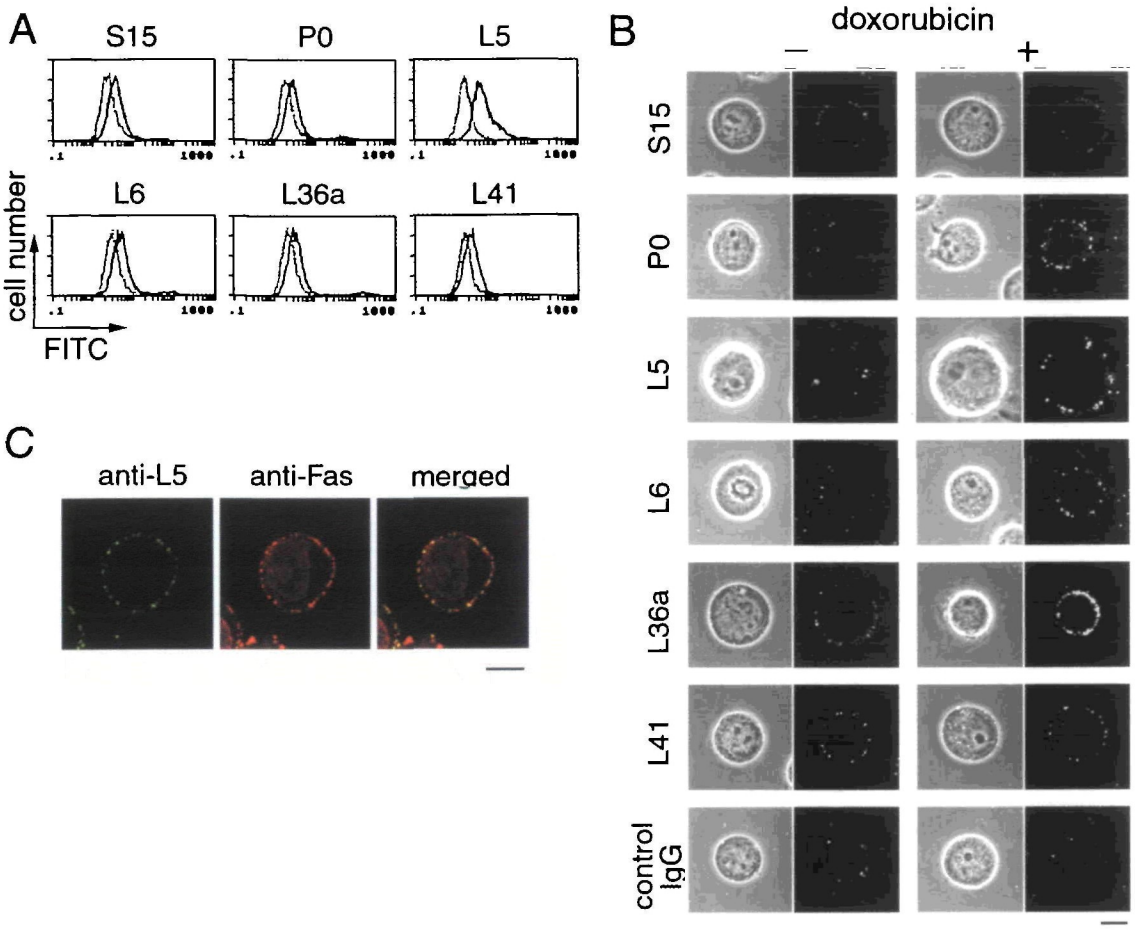

Fig. 8. **Presence of ribosomal proteins at the surface of apoptotic Jurkat cells.** A: Jurkat cells treated with doxorubicin (solid lines) or left untreated (broken lines) were analyzed by flow cytometry with 28 kinds of anti–ribosomal protein antisera. Results for antisera that showed more efficient binding to doxorubicin-treated cells are presented. B: The same cells were subjected to immunofluorescence with the six anti–ribosomal protein IgGs or control normal rabbit IgG. Phase-contrast (left) and fluorescence (right) views of the same fields are shown. Bar = 10 μm. C: Doxorubicin-treated Jurkat cells were simultaneously examined for the presence of L5 and Fas by immunofluorescence. Note that staining of the cell interior in the right two panels is due to fluorescence from DNA-bound doxorubicin. Bar = 10 μm.

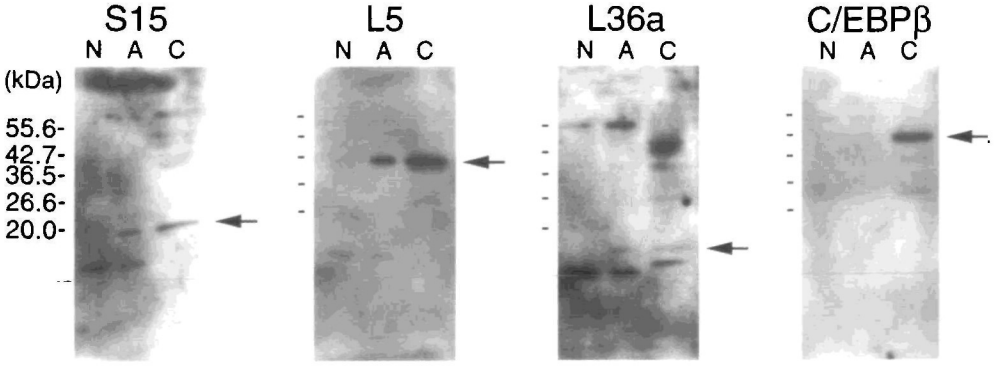

Fig. 9. **Detection of ribosomal proteins in surface proteins of apoptotic Jurkat cells.** Surface proteins of doxorubicin-treated (A) or normal (N) Jurkat cells were analyzed by Western blotting with anti–ribosomal protein antisera or an antibody recognizing the transcription factor C/EBPβ. Lanes "C" contained whole-cell lysates of doxorubicin-treated Jurkat cells as a positive control. The arrows point to the positions of specific signals.

bound to the permeabilized Jurkat cells more intensely than a control rabbit serum in a manner inhibitable by the cognate antigen peptides (data not shown). We thus used these antisera to analyze doxorubicin-treated cells for the surface expression of ribosomal proteins. As depicted in Fig. 8A, six antibodies bound to apoptotic cells more efficiently than to normal cells. To examine the binding of these antibodies to the surface of apoptotic Jurkat cells by another method, the same cells were subjected to immunofluorescence and analyzed by confocal microscopy. Most antibodies, except for two with only a marginal effect, gave signals at the periphery of doxorubicin-treated cells at levels well beyond background (Fig. 8B). Furthermore, the signals with the anti-L5 antibody were entirely included within

those obtained with an antibody recognizing the cell surface protein Fas (Fig. 8C). Finally, we confirmed the surface expression of ribosomal proteins by Western blotting. Surface proteins were isolated from either doxorubicin-treated or untreated Jurkat cells and examined for the presence of S15, L5, and L36a (Fig. 9). All the three ribosomal proteins were detectable only with the surface proteins prepared from apoptotic cells. In contrast, the same protein sample did not give a signal with a control antibody recognizing a protein localized in the nucleus. All the above results collectively indicate that five large subunit proteins, P0, L5, L6, L36a, and L41, and one small subunit protein, S15, are present on the surface of apoptotic Jurkat cells.

DISCUSSION

We analyzed changes in the amount and localization of eukaryotic ribosomal proteins during apoptosis using specific antibodies. Western blotting analyses of 28 ribosomal proteins revealed that three of them, S18, L5, and L14, underwent degradation during apoptosis induced by the anticancer drug doxorubicin or the agonistic anti-Fas monoclonal antibody, and suggested that ribosomes with decreased amounts of particular ribosomal proteins exist in apoptotic cells. It is not likely that these proteins are cleaved directly by caspases since they contain no amino acid sequences that are targeted by caspases. These results are distinct from those previously obtained in the analysis with human breast carcinoma cells, in which the amount of S11 specifically decreased during apoptosis (*16*). Changes in the amount of ribosomal proteins in apoptotic cells are thus likely to be cell-type specific. Moreover, the amount of the 80S ribosome complex decreased in apoptotic cells, suggesting that the formation of the complex was inhibited. Although such deficient ribosomes are most likely not functional in protein synthesis, the above changes in the structure of ribosomes do not seem to be the major cause of the decrease in the rate of protein synthesis during apoptosis, because protein synthesis declined well before the changes in the ribosomal proteins became detectable. In contrast, degradation of the 28S ribosomal RNA paralleled the rate of protein synthesis during apoptosis (*3*). It thus seems that the change in the structure of ribosomal RNA is more important for the inhibition of protein synthesis than changes in ribosomal proteins at early stages of apoptosis. We speculate that the degradation of ribosomal proteins completely inactivates cellular functions at the final step in the process of apoptosis.

Another possible role of the structural changes of ribosomes is regulation of the extra-ribosomal functions of ribosomal proteins. Yamamoto and colleagues have shown that S19, which is dimerized and released from apoptotic cells, functions as a chemoattractant (*14*). They proposed that macrophages are attracted by the modified S19 to sites rich in apoptotic cells. We were unable to verify whether dimerized S19 is released from apoptotic Jurkat cells, since our antiserum raised against the S19 sequence did not react with the native protein. As another possible target function, Casciola-Rosen *et al.* have shown that cellular components including a ribosomal protein translocate to the membrane bleb in apoptotic cells and suggested the possibility that such molecules serve as autoantigens (*10*). Our flow cytometric analyses with 28 kinds of anti–ribosomal protein antibodies suggested that several ribosomal proteins move to the surface of apoptotic cells during apoptosis, and this was confirmed by immunofluorescence and Western blotting analyses. Although the results reported by Casciola-Rosen *et al.* do not necessarily mean the surface expression of the ribosomal protein, our results clearly showed that several proteins exist at the surface of apoptotic Jurkat cells. Any proteins that are normally restricted to the cell interior should readily be detected by the immune surveillance system when they are exposed to the cell surface. A probable consequence of the immune recognition is the phagocytic clearance of cells possessing such externalized molecules. Externalized ribosomal proteins could thus serve as markers for recognition of apoptotic cells by phagocytic cells. Apoptotic cells are rapidly and selectively eliminated from the organism by phagocytosis, and this is defined by specific recognition of target cells by phagocytes (*22*). A variety of molecules, including proteins, phospholipids and sugars, have been proposed to act as phagocytosis markers when expressed on the surface of apoptotic cells (*22*). Phagocytic cells such as macrophages recognize those markers using specific receptors and subsequently engulf apoptotic cells (*22*). There is as yet no evidence that ribosomal proteins act as phagocytosis markers. Experiments are now in progress to examine whether any ribosomal proteins that were detectable on the surface of apoptotic Jurkat cells are involved in the recognition of apoptotic Jurkat cells by macrophages.

REFERENCES

1. Zhou, B.-B., Li, H., Yuan, J., and Kirschner, M.W. (1998) Caspase-dependent activation of cyclin-dependent kinases during Fas-induced apoptosis in Jurkat cells. *Proc. Natl. Acad. Sci. USA* **95**, 6785–6790
2. Marissen, W.E. and Lloyd, R.E. (1998) Eukaryotic translation initiation factor 4G is targeted for proteolytic cleavage by caspase 3 during inhibition of translation in apoptotic cells. *Mol. Cell. Biol.* **18**, 7565–7574
3. Nadano, D. and Sato, T.-A. (2000) Caspase-3-dependent and -independent degradation of 28 S ribosomal RNA may be involved in the inhibition of protein synthesis during apoptosis initiated by death receptor engagement. *J. Biol. Chem.* **275**, 13967–13973
4. Marissen, W.E., Gradi, A., Sonenberg, N., and Lloyd, R.E. (2000) Cleavage of eukaryotic translation initiation factor 4GII correlated with translation inhibition during apoptosis. *Cell Death Differ.* **7**, 1234–1243
5. Houge, G., Robaye, B., Eikhom, T.S., Golstein, J., Mellgren, G., Gjertsen, B.T., Lanotte, M., and Døskeland, S.O. (1995) Fine mapping of 28S rRNA sites specifically cleaved in cells undergoing apoptosis. *Mol. Cell. Biol.* **15**, 2051–2062
6. Lei, H., Furth, E.E., Kalluri, R., Chiou, T., Tilly, K.I., Tilly, J.L., Elkon, K.B., Jeffrey, J.J., and Strauss III, J.F. (1996) A program of cell death and extracellular matrix degradation is activated in the amnion before the onset of labor. *J. Clin. Invest.* **98**, 1971–1978
7. King, K.L., Jewell, C.M., Bortner, C.D., and Cidlowski, J.A. (2000) 28S ribosome degradation in lymphoid cell apoptosis: evidence for caspase and Bcl-2-dependent and -independent pathways *Cell Death Differ.* **7**, 994–1001
8. Bushell, M., Poncet, D., Marissen, W.E., Flotow, H., Lloyd, R.E., Clemens, M.J., and Morley, S.J. (2000) Cleavage of polypeptide chain initiation factor eIF4GI during apoptosis in lymphoma cells: characterization of an internal fragment generated by caspase-3-mediated cleavage. *Cell Death Differ.* **7**, 628–636
9. Clemens, M.J., Bushell, M., Jeffrey, I.W., Pain, V.M., and Morley, S.J. (2000) Translation initiation factor modifications and

the regulation of protein synthesis in apoptotic cells. *Cell Death Differ.* **7**, 603–615
10. Casciola-Rosen, L.A., Anhalt, G., and Rosen, A. (1994) Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J. Exp. Med.* **179**, 1317–1330
11. Horino, K., Nishiura, H., Ohsako, T., Shibuya, Y., Hiraoka, T., Kitamura, N., and Yamamoto, T. (1998) A monocyte chemotactic factor, S19 ribosomal protein dimer, in phagocytic clearance of apoptotic cells. *Lab. Invest.* **78**, 603–617
12. Nishiura, H., Shibuya, Y., and Yamamoto, T. (1998) S19 ribosomal protein cross-linked dimer causes monocyte-predominant infiltration by means of molecular mimicry to complement C5a. *Lab. Invest.* **78**, 1615–1623
13. Nishiura, H., Tanase, S., Sibuya, Y., Nishimura, T., and Yamamoto, T. (1999) Determination of the cross-linked residues in homo-dimerization of S19 ribosomal protein concomitant with exhibition of monocyte chemotactic activity. *Lab. Invest.* **79**, 915–923
14. Yamamoto, T. (2000) Molecular mechanism of monocyte predominant infiltration in chronic inflammation: mediation by a novel monocyte chemotactic factor, S19 ribosomal protein dimer. *Pathol. Int.* **50**, 863–871
15. Brockstedt, E., Rickers, A., Kostka, S., Laubersheimer, A., Dörken, B., Wittmann-Liebold, B., Bommert, K., and Otto, A. (1998) Identification of apoptosis-associated proteins in a human Burkitt lymphoma cell line: cleavage of heterogeneous nuclear ribonucleoprotein A1 by caspase 3. *J. Biol. Chem.* **273**, 28057–28064
16. Nadano, D., Aoki, C., Yoshinaka, T., Irie, S., and Sato, T.-A. (2001) Electrophoretic characterization of ribosomal subunits and proteins in apoptosis: specific downregulation of S11 in staurosporine-treated human breast carcinoma cells. *Biochemistry* **40**, 15184–15193
17. Spaderi, S., Pedarali, N.G., Focher, F., Montecucco, A., Bordoni, T., Geroni, C., Giuliani, F.C., Ventrella, G., Arcomone, F., and Ciarocchi, G. (1986) DNA polymerases and DNA topoisomerases as targets for the development of anticancer drugs. *Anticancer Res.* **6**, 935–940
18. Yonehara, S., Ishii, A., and Yonehara, M. (1989) A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen codownregulated with the receptor of tumor necrosis factor. *J. Exp. Med.* **169**, 1747–1756
19. Shiratsuchi, A. and Nakanishi, Y. (1999) Phosphatidylserine-mediated phagocytosis of anticancer drug-treated cells by macrophages. *J. Biochem.* **126**, 1101–1106
20. Bommer, U.A., Burkhardt, N., Jünemann, R., Spahn, C.M.T., Triana-Alonso, F.J., and Nierhaus, K.H. (1997) Ribosomes and polysomes in *Subcellular Fractionation: A Practical Approach* (Graham, J.M. and Rickwood, D., eds.) pp. 271–301, Oxford University Press, New York
21. Nadano, D., Ishihara, G., Aoki, C., Yoshinaka, T., Irie, S., and Sato, T. (2000) Preparation and characterization of antibodies against human ribosomal proteins: heterogeneous expression of S11 and S30 in a panel of human cancer cell lines. *Jpn. J. Cancer Res.* **91**, 802–810
22. Savill, J. and Fadok, V. (2000) Corpse clearance defines the meaning of cell death. *Nature* **407**, 784–788