The fate of cytosolic proteins was studied during Fas-induced cell death of Jurkat T-lymphocytes by proteome analysis. Among 1000 spots resolved in two-dimensional gels, comparison of control versus apoptotic cells revealed that the signal intensity of 19 spots decreased or even disappeared, whereas 38 novel spots emerged. These proteins were further analyzed with respect to de novo protein synthesis, phosphorylation status, and intracellular localization by metabolic labeling and analysis of subcellular protein fractions in combination with two-dimensional Western blots and mass spectrometry analysis of tryptic digests. We found that e.g. hsp27, hsp70B, calmodulin, and H-ras synthesis was induced upon Fas signaling. 34 proteins were affected by dephosphorylation (e.g. endoplasmic) and phosphorylation (e.g. hsc70, hsp37, and hsp90). Nuclear annexin IV translocated to the cytosol, whereas decreasing cytosolic TCP-1α became detectable in the nucleus. In addition, degradation of 12 proteins was observed; among them myosin heavy chain was identified as a novel caspase target. Fas-induced proteome alterations were compared with those of other cell death inducers, indicating specific physiological characteristics of different cell death mechanisms, consequent to as well as independent of caspase activation. Characteristic proteome alterations of apoptotic cells at early time points were found reminiscent of those of malignant cells in vivo.

Apoptosis represents a genetically programmed suicide process indispensable for the life of higher organisms. Aberrations of apoptotic mechanisms were causally implicated in severe diseases such as cancer (1, 2). Because apoptosis was characterized by morphological features such as membrane blebbing, cell shrinkage, chromatin condensation, and formation of apoptotic bodies (3), it became evident that various proteins are affected during this process. The specific proteolytic activities of caspases, cysteiny1-aspartate proteases normally expressed as latent zymogens and activated during apoptosis, were recognized as responsible for many of these morphologic alterations (4, 5). Signaling pathways, beginning with the activation of surface receptors ultimately resulting in the activation of caspases and hence the cellular demise, have been well characterized (6). However, several proteins became known to potentially inhibit (7) or promote (8, 9) the onset of apoptosis by different means. Malignant diseases have been characterized by the imbalance of proliferation and cell death, resulting in a net gain of tumor mass (10, 11), with regulatory cross-talk between these processes being evident (12). During malignant transformation of cells, resistance to apoptosis may develop by the expression of antiapoptotic proteins (13), thus giving spreading tumor cells a higher chance to develop metastasis (14).

In this study we addressed the question whether cells induced to undergo apoptosis would solely follow the cell death program or alternatively employ additional mechanisms unrelated with caspase activation. Potential intrinsic antiapoptotic responses could help explain how cells proceed during the establishment of resistance to apoptosis leading to diseases. To gain a broad overview of cell activities upon apoptosis induction, we performed a comprehensive proteome analysis to study Fas-mediated apoptosis in Jurkat cells. This cell system was chosen because much is known about the cell death initiation mechanisms (6), because the onset of apoptosis occurs in a quite synchronous manner, and because virtually all cells are committed to cell death and finally die, as described previously (15). To observe specificities with respect to the cell death induction mechanism, the data were compared with those obtained with staurosporine, camptothecin, and oligomycin.

The totality of proteins expressed from the genome of a cell is referred to as proteome (16). The proteome is highly dynamic and depends on many different parameters affecting cells. Proteome analysis may be performed by high resolution 2D1 gel electrophoresis, separating proteins according to their molecular weight and electric charge, which yields highly reproducible and characteristic 2D protein patterns (17). Several studies have already focused on apoptosis-related proteins in apoptotic cells (18–21). In this study we investigated whether proteome alterations during apoptosis originated from new protein synthesis, protein translocation, or the formation of new protein isoforms or degradation products. The focus was on cytosolic proteins; data obtained with regard to other protein fractions will be presented elsewhere.

57 of more than 1000 protein spots resolved were found to be altered during Fas-induced apoptosis. Synthesis of hsp70B, for example, was newly but transiently induced. The translation rate of several signal transduction and cytoskeletal proteins was significantly increased. Phosphorylation of some chaperones and signaling proteins was accompanied by the dephosphorylation of others. Caspase-mediated protein degradation

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1 The abbreviations used are: 2D, two-dimensional; hsp, heat shock protein; mAb, monoclonal antibody; FCS, fetal calf serum; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; aa, amino acid; z-VAD-fmk, benzoyloxycarbonyl-Val-Ala-Asp(methoxy)fluoromethyl ketone; hsc, heat shock cognate.
was evidenced by different means for 12 proteins. Investigation of proteome alterations using other apoptosis inducers revealed that the same proteins were cleaved, whereas protein translation and (de)phosphorylation profiles differed somewhat. Necrosis displayed few similarities with apoptosis, as well as calpain-mediated protein degradation and a potential stress response. This study demonstrates that many data on a large number of proteins may be obtained by high throughput proteome analysis, providing new insights into the fate of cells during apoptosis.

MATERIALS AND METHODS

Antibodies—Anti-caspase 3 polyclonal IgG and anti-Fas (CD95) monoclonal antibody (mAb) (Clone CH-11) was purchased from Upstate Biotechnology (Lake Placid, NY); anti-hsp27 mAb (Clone G3.1) was from Neomarkers (Fremont, CA); anti-hsp60 mAb (Clone LR-1) and anti-hsp57 (FKBP59) mAb (SRA-I400) was from StressGen (Victoria, Canada). Anti-hsp70 mAb (Clone W27) was purchased from Neomarkers; anti-hsp90 mAb (Clone 3G3) was from Alexis (San Diego, CA); anti-gelsolin mAb (Clone GS-2C4) was from Sigma (Sigma); anti-Rho-A mAb (25C4) was from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-H-ras mAb (M0637) was from Dako (Carpinteria, CA).

Induction of Apoptosis—Jurkat cells were routinely cultivated in RPMI 1640 supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere containing 5% CO₂. For induction of apoptosis, Jurkat cells were washed in serum-free medium and re-seeded at a density of 10⁵ cells/ml in medium containing 1% FCS. Apoptosis was induced in Jurkat cells 15 min thereafter by addition of either anti-Fas (CD95) antibody (50 ng/ml) camptothecin (Sigma) to cellular media or by 10 μM staurosporine. As demonstrated previously (15), virtually all of the cells were committed to die by apoptosis; the apoptotic index was at least 90% and was not affected by a 24-hr treatment. At this time, cells displayed an apoptotic index of about 50%, whereas the overall translation rate was found to be only slightly affected. This time was chosen to allow potential transcriptional regulations to become manifest at the protein level. Indeed, we were unable to recover vital cells when we repeatedly washed the cells with fresh medium 3 h after addition of antibody (data not shown).

RESULTS

Jurkat cells were induced to undergo apoptosis by treatment with anti-mCD95 antibody. As demonstrated previously (15), virtually all of the cells were committed to die by apoptosis; almost 100% chromatin condensation was observed after 12 h. Indeed, we were unable to recover vital cells when we repeatedly washed the cells with fresh medium 3 h after addition of antibody (data not shown).

Proteome analysis was performed with cells 5 h after antibody treatment. At this time, cells displayed an apoptotic index of about 50%, whereas the overall translation rate was found to be only slightly affected. This time was chosen to allow potential transcriptional regulations to become manifest at the protein level. Indeed, translational up-regulations became evident at that time that were no longer detectable at later time points (see Table II). Cytosol was isolated from control and apoptotic cell populations by analysis of high resolution two-dimensional gel electrophoresis. Computer-assisted quantitative analysis of the respective silver-stained spot patterns revealed 19 spots with decreasing intensity (marked by hexagons in Figs. 1 and 2), 38 new spots or spots with strongly increased intensity (marked by circles in Fig. 2), and more than 1000 spots that apparently remained unaffected (Figs. 1 and 2; see
Table II). These data were reproduced in two additional independent experiments. The decrease of protein spot intensities was rather slight at 5 h after anti-Fas treatment but much more pronounced at 8 and 12 h after apoptosis induction (see Table II). The affected proteins were investigated by 2D Western blotting as described previously (26) and mass spectrometry of tryptic digests, resulting in the identification of those listed in Table I. Further experiments were performed to deduce whether the altered spots were due to protein synthesis, modification, translocation, or degradation. In addition, the proteome alteration profile of Fas-induced cells was compared with that of staurosporine-, camptothecin-, and oligomycin-induced cells.

Protein Synthesis—Control and apoptotic Jurkat cells were metabolically labeled for 2 h with a mixture of [35S]Met and [35S]Cys, beginning 3 h after antibody treatment. The resulting, previously silver-stained 2D gels were autoradiographed, which allowed us to record relative 35S incorporation rates for each protein resolved. Comparison of the spot intensities of control and apoptotic samples, considering the respective silver-stained spot intensities, gave a measure for changes of translation rates during apoptosis. As expected, the 35S incorporation rates of those proteins degraded during apoptosis were found to be decreased. Calmodulin, gelsolin, kinesin
Fas-induced Proteome Alterations

TABLE I
List of identified protein spots

| Protein name | Swiss-Prot. accession number | Identified by |
|--------------|------------------------------|--------------|
| Alpha-actin 1, cytoskeletal isoform | P12814 | M(11) |
| Apopain (caspase 3) | P17066 | W |
| Eukaryotic translation initiation factor 4 gamma (EIF-4G) | P24534 | M(9), P |
| Importin beta-3 | O00410 | M(9) |
| Heat shock protein hsp 90-alpha (hsp90) | P25963 | M(14), P |
| Heat-shock protein 105 kDa (hsp110) | P25965 | M(16), P |
| Major histocompatibility complex enhancer-binding protein mad3 (IxBa) | P25967 | M(14) |
| Myo sin light chain alkali, non-muscle isoform | P16475 | M(12) |
| Protein disulfide isomerase | P07237 | P |
| Protein phosphatase pp2a, 65-kDa regulatory subunit, alpha isoform | P30153 | M(13) |
| rho GDP-dissociation inhibitor 2 (rho GDI 2) | P55666 | M(16), P |
| T-complex protein 1, alpha subunit (TCP-1a) | P17987 | M(10), P |
| Tropomyosin, cytoskeletal type (TM30-NM) | P12524 | M(12), P |

Proteins with decreasing spot intensity during apoptosis

| Protein name | Swiss-Prot. accession number | Identified by |
|--------------|------------------------------|--------------|
| Actin, cytoplasmic 1, fragment | P02550 | M(9) |
| Alpha-actin 1, cytoskeletal isoform, modified | P12814 | M(11) |
| Annexin IV | P08925 | M(12) |
| Elongation factor 1-beta (EF-1a) | P24534 | M(9), P |
| Endoplasm | P14625 | M(14), P |
| Heat shock 27-kDa protein (hsp27) | P17066 | P |
| Heat shock 70-kDa protein 6 (hsp70B') | P17066 | P |
| Myo sin heavy chain, nonmuscle type A', fragment | P25579 | M(10) |
| Myo sin light chain alkali, non-muscle isoform, phosphorylated | P16475 | M(12) |
| rho GDP-dissociation inhibitor 1, phosphorylated | P52565 | P |
| rho GDP-dissociation inhibitor 2, fragment | P52566 | M(16) |
| rho GDP-dissociation inhibitor 2, fragment | P52566 | M(14) |
| Spectrin alpha chain (fodrin), fragment 150 kDa | Q13813 | M(38) |
| Spectrin alpha chain (fodrin), fragment 120 kDa | Q13813 | M(34) |
| Transforming protein p21/H-ras-1 | Q13813 | M(38) |
| Tubulin alpha-4 chain, putative fragment | P05215 | M(15) |
| Vimentin, fragment | P08870 | M(7) |

Protein Translocation—Protein spots disappearing or appearing as new in the cytosol fraction of apoptotic cells were assessed with respect to a potential change in localization. Their occurrence in cytosol pellet and nuclear protein fractions of control and apoptotic cells was investigated by comparison of the respective 2D spot patterns. Protein spots present in all fractions compared, such as some chaperone proteins (30), served as internal anchors for correct pattern overlay. Proteins newly appearing in the cytosol of apoptotic cells but present, for example, in the nuclear fraction of control cells were regarded as translocating. Translocation of nuclear annexin IV to the cytosol is evidenced in Fig. 3C. The presently observed translocation of yet unknown proteins at 92 kDa/pI 4.7 and 95 kDa/pI 4.6 (Table II) might correspond to data published by Ma et al. (31). Translocation from the cytosol to the nucleus is evidenced in the case of TCP-1a in Fig. 3C.

Protein Degradation—Several observations made by proteome analysis were indicative of protein degradation. First of all, several proteins were found to decrease or disappear during apoptosis; among others these included caspases 3 and 8, gelolin, importin beta-3, myosin light chain, protein phosphatase 2A, rho GDI 2, and vimentin. However, some of these, including hsp90, hsp110, protein disulfide isomerase, and tropomyosin, were found to be accumulated in the cytosol pellet fraction of apoptotic cells and were therefore not considered as degraded, but rather translocated or aggregated. Secondly, several spots appearing as new during apoptosis were found to represent degradation products. Interestingly, these spots displayed rather few 35S labels with regard to the respective silver stain intensities (see Fig. 5, D versus B), in contrast to new protein spots emerging because of protein modification or synthesis.
Figs. 3. Protein synthesis, phosphorylation, and translocation during apoptosis evidenced by the comparison of the respective gel sections. If not indicated otherwise, the protein samples referred to as control and Fas correspond to those of Fig. 1 and Fig. 2, respectively. A, induced protein synthesis evidenced by \(^{35}\)S autoradiography and silver staining. After silver staining, gels were equilibrated with Enlightning, dried, and exposed to x-ray films. The \(^{35}\)S label corresponds to the translaton rate of the respective protein. Note that the increased translation of calmodulin is not reflected at the silver stain level and that hsp27 is not detectable in control cells. B, (de)phosphorylation evidenced by \(^{32}\)P autoradiography and silver staining. \(^{32}\)P labeling was performed analogous to \(^{35}\)S labeling, utilizing phosphate-free medium and 2.5 mCi/10^7 cells \(^{32}\)Porthophosphoric acid. After silver staining, gels were dried and exposed to x-ray films. Two isoforms of the indicated proteins are encircled, the more acidic isoform labeled by autoradiography, hence phosphorylated, and the basic isoform unphosphorylated, not labeled, and observed only by silver staining. Note that dephosphorylation of endoplasm in is indicated by a decrease of the \(^{32}\)P label and a more prominent appearance of the basic, dephosphorylated isoform by silver staining. Phosphorylation of heat shock cognate 70 (hsc70) is indicated by the increased \(^{32}\)P label and a more prominent appearance of the more acidic, phosphorylated isoform by silver staining. C, translocation evidenced by comparison of subcellular fractions. Cytosol was obtained by the lysis of cells in 0.05% Nonidet P-40 in hypotonic buffer, pelleting of nuclei, and ultracentrifugation of the resulting supernatant at 100,000 \( \times g \) for 60 min; the pellet corresponds to the cytoplasmic pellet. The nuclear matrix protein fraction was prepared as described in detail (23). Whereas annexin IV appeared as new in the cytosol during apoptosis, it disappeared from the nuclear matrix protein fraction. On the other hand, TCP-1a decreased in the cytosol during apoptosis, concomitantly becoming more prominent in the nuclear matrix fraction. During chromat condensation, the nuclear matrix fraction displayed various other spot alterations that will be described in detail elsewhere.
### Table II
Comparison of protein alterations with respect to death signal, metabolic label, and subcellular distribution

(−), phosphorylated isoform; *, degradation product of; Fas, anti-CD95 antibody-treated; STS, staurosporine-treated; OM, oligomycin-treated (for the indicated time); con, control; =, in comparison with; +, spot present; ++, very high intensity; ++++, very high intensity; −, intensity not significantly changed; <, decreased intensity; >, increased intensity; AR, autoradiography; CYTP, cytosol pellet; NM, nuclear matrix; Apo, apoptosis; Phospho., phosphorylation; Degrad., degradation; prod., product; Syn., synthesis; Mod., modification; −, spot absent.

#### Proteins with decreasing spot intensity during apoptosis

| Protein | Con | Fas (5 h) | Fas (12 h) | STS (6 h) | OM (5 h) | 35S AR | 32P AR | CYTP | NM | Interpretation |
|---------|-----|-----------|------------|-----------|----------|--------|--------|------|----|----------------|
| α-Actinin | ++ | + | − | < | = | < | − | ++ | + | − | +/− | Modification |
| Caspase-3 | ++ | + | − | < | = | < | − | − | − | + | +/− | Processing |
| Caspase-8 | + | + | + | < | = | < | − | − | − | − | − | −/− | Processing |
| EF-1β | ++ | + | < | < | < | = | − | + | + | − | − | − | Degradation |
| EF-4G | + | + | − | < | < | < | − | + | + | − | − | − | Degradation |
| (p-)Endoplasm | ++ | + | < | < | < | < | ≤ | − | − | − | − | − | Desphosphorylation |
| hsp90 | ++ | + | < | < | < | < | − | ≥ | + | + | − | + | Aggregation |
| hsp110 | ++ | + | < | < | < | < | ≤ | − | − | − | − | + | Aggregation |
| Ino-B-α | ++ | + | < | < | < | < | ≤ | − | − | − | − | + | Degradation |
| Importin-β3 | + | + | − | < | < | < | ≤ | − | − | − | − | + | Desphosph./Degrad. |
| Myosin Ic | ++ | + | < | < | < | < | ≤ | − | − | − | − | + | Degradation |
| p19/6.2 | ++ | + | < | < | < | < | ≤ | − | − | − | − | + | Dephosphorylation |
| p32/5.9 | ++ | + | < | < | < | < | ≤ | − | − | − | − | + | Dephosphorylation |
| p70/7.1 | + | + | < | < | < | < | ≤ | − | − | − | − | + | Synthesis |
| PDI | ++ | + | < | < | < | < | ≤ | − | − | − | − | + | Aggregation |
| PPI | ++ | + | < | < | < | < | ≤ | − | − | − | − | + | Aggregation |
| rho GDI 2 | ++ | + | < | < | < | < | ≤ | − | − | − | − | + | Degradation |
| TCP-1α | ++ | + | < | < | < | < | ≤ | − | − | − | − | + | Translocation |
| TM30-NM | ++ | + | < | < | < | < | ≤ | − | − | − | − | + | Degrad./Aggregation |

#### New protein spots emerging during apoptosis

| Protein | Con | Fas (5 h) | Fas (12 h) | STS (6 h) | OM (5 h) | 35S AR | 32P AR | CYTP | NM | Interpretation |
|---------|-----|-----------|------------|-----------|----------|--------|--------|------|----|----------------|
| α-Actinin modified | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Modification |
| *Actin | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Cleavage prod. |
| *Caspase-3 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| (p-)IEF-1β | + | + | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| Endoplasm | + | + | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| hsp27 | + | + | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| (p-)hsp27 | + | + | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| hsp70-6 | − | − | − | − | − | − | − | − | − | − | − | − | − | − | −/− | Phosphorylation |
| H-ras | − | − | − | − | − | − | − | − | − | − | − | − | − | − | −/− | Phosphorylation |
| (p-)Ino-B-α | − | − | − | − | − | − | − | − | − | − | − | − | − | − | −/− | Phosphorylation |
| (p-)Myosin Ic | − | − | − | − | − | − | − | − | − | − | − | − | − | − | −/− | Phosphorylation |
| *NMIMIC-A | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p16/6.1 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p18/4.4 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p23/6.6 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p28/6.6 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p32/5.7 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p39/5.0 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p41/4.6 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p42/4.4 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p44/4.2 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p49/5.1 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p52/6.0 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p54/5.8 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p54/5.9 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p57/6.2 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p80/4.9 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p85/4.8 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p92/4.7 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| p95/4.6 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| RFC-36 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| (p-βh GDI 1 | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| *rh GDI 2.2 kDa | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| *rh GDI 2.2 kDa | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| *Spectrin 150 kDa | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| *Spectrin 120 kDa | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| *α-Tubulin | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |
| *Vimentin | − | − | + | + | + | + | + | − | − | − | − | − | − | −/− | Phosphorylation |

47.9 kDa/pI 5.42 for a fragment spanning aa 1–431.

**Protein Alterations Dependent on the Mode of Cell Death Induction**—To discriminate proteins altered specifically during induction of the Fas signaling pathway, we compared the respective protein alterations to those of staurosporine- and camptothecin-treated apoptotic Jurkat cells (Fig. 5, L and M; Table II). No differences were observed with respect to protein degradation products potentially generated by caspase activi-
Cytoskeletal proteins and chaperones are strongly affected during Fas-induced apoptosis

Readout of the current proteome database with respect to two related protein families. Annotation as in Tables I and II. nd, not detectable; AR, autoradiography; W, 2D Western blotting; M, mass spectrometry analysis of tryptic peptides; P, position in 2D gels in relation to published 2D data bases; A, amino acid sequencing; -, intensity not significantly changed; +, spot present; , spot absent; >, increased intensity; <, decreased intensity; Fas, anti-CD95 antibody-treated; con, control; vs. indicated.

| Protein Identified by | degradation evident |
|-----------------------|---------------------|
| Cytoskeletal proteins |                     |
| b-Actin               | W(*), M =            |
| Ezrin                 | M =                 |
| a-Fodrin              | M(**) nd +(***) Yes  |
| Gelsolin              | W >                 |
| Kinesin heavy chain   | M >                 |
| a-Tubulin             | P =                 |
| Vimentin              | W(*) >              |
| rho-A                 | W =                 |
| rho GDI-1             | M =                 |
| rho GDI-2             | M =                 |
| TM30-NM               | M =                 |
| Chaperones            |                     |
| BiP (grp78)           | M >                 |
| Calreticulin          | A(*) =              |
| Calmodulin            | P >                 |
| Endoplasmin           | M >                 |
| ER-60                 | A(*) =              |
| hsc70                 | A(*) =              |
| has27                 | W =                 |
| has57                 | W >                 |
| has60                 | W(*) =              |
| has70                 | W, M(*) =           |
| has70B                | P >                 |
| has90                 | W =                 |
| PDI                   | P(*) =              |
| TCP-1a                | M =                 |
| TCP-18                | M =                 |

FIG. 4. Degradation of myosin heavy chain during apoptosis evidenced by the detection of degradation products. A, gel sections of cytosol and cytosol pellet fractions of control and apoptotic cells as indicated. Circles, positions of the N-terminal fragments; hexagons, positions of the C-terminal fragments. B, mass spectrometry fingerprint of the C-terminal fragment. Coomassie Blue-stained proteins were directly cut out of preparative gels. Matrix-assisted laser desorption ionization-time-of-flight was performed of tryptic protein hydrolysates thereof. C, amino acid sequence of myosin heavy chain; the peptides covered by mass spectrometry of the C-terminal fragment are capitalized and underlined.

Table III

Fas-induced Proteome Alterations

Some data presented so far were selected for the most significant alterations observed. However, all variables, such as relative protein amount (determined by silver staining), translation rate (35S label), and phosphorylation state (32P label), were recorded for each detectable protein of the different subcellular fractions analyzed. Considering different time points and different cell death induction agents as well, finally a multidimensional set of data was obtained. Related gel sections, representing a small part of the final generated data base, are illustrated in Fig. 5. Proteins allocated in the 2D system may be searched by this means for apoptosis-dependent alterations. This approach, i.e., searching for alterations of selected proteins of interest, is exemplified in the case of two protein families, cytoskeletal proteins and chaperones, which appeared to be specific for Fas-induced apoptosis.

On the other hand, the proteome alteration profile of oligomycin-treated, thus necrotic, Jurkat cells was analyzed (Fig. 5K, Table II). As expected, no caspase-mediated degradation events were observed; however, the calpain-generated 150-kDa fodrin fragment (34) was readily identified in necrotic Jurkat cells (Fig. 5K), indicating calpain activity during necrosis. The same fragment was the only degradation product observed in Fas-induced Jurkat cells pretreated with caspase inhibitor z-VAD-fmk (data not shown), confirming that no caspase generated this fragment. The most apparent modifications during necrosis, such as phosphorylation of IkB-a, myosin light chain, and elongation factor 1b or dephosphorylation of endoplasmin, were observed as during apoptotic cell death. Interestingly, hsp90 and hsp110 were again found to be reduced in the cytosol of necrotic cells.

Establishment of a Data Base by Integration of Proteome Analysis Data—As exemplified above, proteins may be affected during apoptosis by quite different means, with different kinetics, and be dependent on the cell death induction pathway. Some data presented so far were selected for the most significantly altered characteristics observed. However, all variables, such as relative protein amount (determined by silver staining), translation rate (35S label), and phosphorylation state (32P label), were recorded for each detectable protein of the different subcellular fractions analyzed. Considering different time points and different cell death induction agents as well, finally a multidimensional set of data was obtained. Related gel sections, representing a small part of the final generated data base, are illustrated in Fig. 5. Proteins allocated in the 2D system may be searched by this means for apoptosis-dependent alterations. This approach, i.e., searching for alterations of selected proteins of interest, is exemplified in the case of two protein families, cytoskeletal proteins and chaperones, which
To sum up, our proteome analysis data suggest that these two regulated, and two were found to be newly induced (Table III). Be dephosphorylated, two were found to be translationally up-regulated. On the other hand, no degradation of chaperone proteins became evident in our system; still three members were found to be phosphorylated, two were found to be dephosphorylated, two were found to be translationally up-regulated, and two were found to be newly induced (Table III). To sum up, our proteome analysis data suggest that these two protein families represent key players during active cell death.

**DISCUSSION**

It was the aim of this study to gain more comprehensive insights into the cellular mechanisms activated during apoptosis by using the proteome analysis. The proteome of a cell is the collection of all proteins expressed by the cell at a particular time. It is a dynamic representation of the cell's state and reflects the cell's metabolic processes, signaling pathways, and response to external stimuli. The proteome of a cell is highly dependent on the conditions to which the cell is exposed and may respond in a quite complex manner. Upon Fas induction, 57 protein spots displayed significant and reproducible alterations, whereas more than 1000 cytosolic protein spots were apparently unaffected in silver-stained 2D gels. This gives an estimate of around 5% of the cytosolic proteins affected during apoptosis. We registered new synthesis of, for example, hsp27 and hsp70; increased translation of, for example, calmodulin, H-ras, and vimentin; phosphorylation of, for example, IcB-α, hsc70, hs90, and myosin light chain; dephosphorylation of, for example, endoplasmin, kinesin heavy chain, and vimentin; and translocation of TCP-1a and annexin IV. In addition, Proteins below silver stain sensitivity displayed altered phosphorylation rates as detected by 32P autoradiography. Some effects were found to be transient and/or specific with respect to the cell death induction pathways, such as the induction of hsp27 and hsp70 and the up-regulation of H-ras, respectively. Experimental data from spot intensities of selected protein fractions, at different time points after induction of apoptosis and with different apoptosis inducers, have been collected in a database, which might enable new mechanistic insights.

The best characterized consequence of apoptosis induction so far, i.e., the activation of caspases, became evident by the detection of an active caspase 3 isoform (Fig. 2) and the disappearance of the procaspases 3 and 8 (Fig. 1). Consequent to the caspase activation was the disappearance of eight proteins (besides the procaspases) and the appearance of 17 degradation products, which was not observed when the caspase inhibitor z-VAD-fmk was applied. Most protein degradations, such as those of actin, fodrin, gelsolin, protein phosphatase 2A, and rho GDI 2, have been described recently (35); however, we also observed the degradation of myosin heavy chain and obtained the first evidence for the degradation of tubulin α. Degradation of cytoskeletal and cytoskeleton-regulating proteins was found to be responsible for most of the prominent spot alterations detected by silver staining.

A stress response of dying cells is reflected by another set of prominent alterations, such as induced synthesis of calmodulin and hsp27, phosphorylation of IcB-α and myosin light chain (36), and dephosphorylation of endoplasmin (37). The decrease of cytosolic hsp90 and hsp110 might be due partially to aggregation caused by binding to non-native proteins emerging during cell death, because they became more prominent in other cell fractions during apoptosis (data not shown). Besides induced synthesis, these stress-related alterations were also observed during necrosis. Because no caspases are activated during necrosis, this is considered to be a caspase-independent cellular response upon induction of cell death, which might be a consequence of a rise in intracellular Ca2+ signaling (38) or the generation of non-native proteins.

The broad spectrum of protein alterations observed during apoptosis might justify some speculations upon further biological implications of cell death signaling. Stress-induced decrease of cytosolic hsp90 has been demonstrated to be able to uncover cryptic genetic variations, leading to the expression of new traits in Drosophila (39). hsp90 decreased strikingly during cell death, with the most acid isoform becoming undetectable after 12 h (data not shown). This observation might indicate the onset of microevolution upon induction of cell death. Cell death has been demonstrated to occur in malignant tumors in addition to cell proliferation (11). Considering a great number of affected cells and a prolonged time period, cell death signaling might be considered as a Darwinian driving force to select for cell death incompetence, which is a common feature of many malignant cells (7, 40). Several of our data would support this interpretation. hsp27, which was presently found to be induced by anti-Fas treatment, has been described to inhibit the mitochondria-mediated apoptosis induction pathway (41). In addition, some kinds of tumor cells have been demonstrated to exhibit high levels of hsp27 (42). Highly expressed H-ras, one of the most common features of cancer cells, may have positive...
or negative effects on cell growth, differentiation, and death during multistage carcinogenesis (43). H-ras was found to be induced during Fas-induced apoptosis and might represent another antiapoptotic response mediated by Akt kinase, consequent to the activation of PI3-kinase or NF-κB (44). The presently observed phosphorylation of IκB-α (Table II) would be compatible with a transient activation of NF-κB. In addition, some antiapoptotic cell responses might also be indicated by the induction of calmodulin, a slight induction of grp78, activation of endoplasmin by dephosphorylation (45), and phosphorylation of hsc70. Calmodulin signaling was reported to activate downstream Akt kinase (46), thereby exerting antiapoptotic activity (44). hsc70 has been demonstrated to represent a target for tyrosine kinases (47) and to cooperate with BAG-1 in mediating antiapoptotic activities (48). These data show that transient events observed in cells on their way to cell death are similar to those established in malignant cells. Thus, our data may indicate that chronic cell death induction, which never results in death in virtually all cells affected in vivo, might be considered as a driving force for the formation of cell-death-incompetent cells, paving the way for the formation of malignant tumors.

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REFERENCES
1. Steller, H. (1995) Science 267, 1445–1449
2. Evan, G. (1997) Int. J. Cancer 71, 709–711
3. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) Br. J. Cancer 26, 239–257
4. Martin, S. J., and Green, D. R. (1995) Cell 82, 349–352
5. Thornberry, N. A., and Rano, T. A. (1998) Oncogene 17, 3331–3340
6. Leist, M., Single, B., Castoldi, A. F., Kuhnle, S., and Nicotera, P. (1997) J. Biol. Chem. 272, 17907–17911
7. Sculte-Hermann, R., Bursch, W., Low-Baselli, A., Wagner, A., and Grasl-Kraupp, B. (1997) Cell Biol. Toxicol. 13, 339–348
8. Schulte-Hermann, R., Bursch, W., Low-Baselli, A., Wagner, A., and Grasl-Kraupp, B. (1999) IARC Int. Agency Res. Cancer Sci. Publ. 146, 273–285
9. Gloe, M., and Bruce, A. H. (1999) Curr. Opin. Cell Biol. 11, 745–752
10. LaCasse, E. C., Baird, S., Korneluk, R. G., and MacKenzie, A. E. (1998) Oncogene 17, 3247–3259
11. Gliksy, V. V. (1996) Cancer Lett. 101, 43–51
12. Gotzmann, J., Meissner, M., and Gerber, C. (2000) Cell Death Differ. 7, 425–438
13. Anderson, N. L., and Anderson, N. G. (1998) Electrophoresis 19, 1853–1861
14. Carriuolo-Marlagne, C., Dessi, F., and Ben-Ari, Y. (1996) Electrophoresis 17, 1718–1786
15. Bockisch, E., Rickers, A., Kostka, S., Lauberhofer, A., Dorken, B., Wittmann-Liebold, B., Bommer, K., and Otto, A. (1998) J. Biol. Chem. 273, 28057–28064
16. Gerner, C., Seedorf, C., and Sauerermann, G. (1998) Exp. Cell Res. 238, 472–480
17. Prasad, S. C., Soldatenkov, V. A., Kuettel, M. R., Thraves, P. J., Zou, X., and Dritschilo, A. (1999) Electrophoresis 20, 1065–1074
18. Leist, M., Single, B., Castoldi, A. F., Kuhnle, S., and Nicotera, P. (1997) J. Exp. Med. 185, 1481–1486
19. Gerner, C., Holzmann, K., Grimm, R., and Sauerermann, G. (1998) J. Cell Biol. 71, 363–374
20. Hochstrasser, D. F., Harrington, M. G., Hochstrasser, A. C., Miller, M. J., and Cherli, C. R. (1988) Anal. Biochem. 173, 424–435
21. Wray, N., Boulikas, T., Wray, V., and Hancock, R. (1981) Anal. Biochem. 118, 197–203
22. Gotzmann, J., and Gerner, C. (1999) Electrophoresis 21, 523–525
23. Fountoulakis, M., and Langen, H. (1997) Anal. Biochem. 250, 153–156
24. Wilkins, M. R., and Williams, K. L. (1997) J. Theor. Biol. 186, 7–15
25. Bjellqvist, B., Hughes, G. J., Pasquali, Ch., Paquet, N., Ravier, F., Sanchez, J.-Ch., Frutiger, S., and Hochstrasser, D. F. (1993) Electrophoresis 14, 1025–1031
26. Gerner, C., Holzmann, K., Meissner, M., Gotzmann, J., Grimm, R., and Sauerermann, G. (1999) J. Cell. Biol. 72, 145–151
27. Ma, H., Siegel, A. J., and Benezra, R. (1999) J. Cell Biol. 146, 531–542
28. Na, S., Chuang, T. H., Cunningham, A., Turi, T. G., Hanke, J. H., Bokoch, G. M., and Danley, D. E. (1996) J. Biol. Chem. 271, 11209–11213
29. Thornberry, N. A., Rano, T. A., Petersen, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., and Nicholson, D. W. (1997) J. Biol. Chem. 272, 17907–17911
30. Waterhouse, N. J., Finucane, D. M., Green, D. R., Ele, J. S., Kumar, S., Almner, E. S., Litwack, G., Khanna, K., Lavin, M. F., and Watters, D. J. (1998) Cell Death Differ. 5, 1051–1061
31. Streh, C., and Schulze-Osthoff, K. (1998) Cell Death Differ. 5, 997–1000
32. Mills, J., Stone, N. L., Erhardt, J., and Pittman, R. N. (1998) J. Cell. Biol. 140, 627–636
33. Csermely, P., Miyata, Y., Schnaider, T., and Yahara, I. (1995) J. Biol. Chem. 270, 6381–6388
34. Sculock, A. B., Horsman, C. D., Bird, G. S. J., Putney, J. W., Jr., and Cidlowski, J. A. (2000) J. Biol. Chem. 275, 30586–30596
35. Rutherford, S. L., and Lindquist, S. (1998) Nature 396, 336–342
36. Hanahan, D., Weinberg, R. A. (2000) Cell 100, 57–70
37. Garrido, C., Bruey, J. M., Frementin, A., Hammond, A., Arrigo, A. P., and Solary, E. (1999) FASEB J. 13, 2061–2070
38. Uozaki, H., Horiuchi, H., Ishida, T., Iijima, T., Imamura, T., and Machinami, R. (1997) Cancer 79, 2336–2344
39. Frame, S., and Balmann, A. (2000) Curr. Opin. Genet. Dev. 10, 106–113
40. Downward, J. (1998) Curr. Opin. Genet. Dev. 8, 49–54
41. McCormick, T. S., McColl, K. S., and Distelhorst, C. W. (1997) J. Biol. Chem. 272, 9087–9092
42. Soderling, T. R. (1999) Trends Biochem. Sci. 24, 232–236
43. Egerton, M., Moritz, R. L., Druker, B., Kelso, A., and Simpson, R. J. (1996) Biochem. Cell Biol. 74, 666–674
44. Takayama, S., Krajewska, S., Krajewska, M., Kitada, S., Zapa, J. M., Kochel, K., Knee, D., Scudiero, D., Tudor, G., Miller, G. J., Miyashita, T., Yamada, M., and Reed, J. C. (1998) Cancer Res. 58, 3116–3131