GDP dissociation inhibitor D4-GDI (Rho-GDI 2), but not the homologous Rho-GDI 1, is cleaved by caspase-3 during drug-induced apoptosis

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Different cytotoxic drugs induce cell death by activating the apoptotic programme; a family of cysteinyl aspartate proteases named caspases has been shown to be involved in the initiation as well as the execution of this kind of cell death. In the present study, cleavage of D4-GDI (Rho-GDI 2), an abundant haemopoietic-cell GDP dissociation inhibitor for the Ras-related Rho family GTPases, was demonstrated after treatment of BJAB Burkitt-like lymphoma cells with taxol or epirubicin. The cleavage of D4-GDI occurred simultaneously with the activation of caspase-3 but preceded DNA fragmentation and the morphological changes associated with apoptotic cell death. By using high-resolution two-dimensional gel electrophoresis it was shown that this cleavage is specific: whereas the level of the homologous protein Rho-GDI 1 was not significantly altered during drug-induced apoptosis and in cytochrome *c*/dATP-activated cellular

extracts, D4-GDI disappeared owing to proteolytic cleavage. Inhibitor experiments with Z-DEVD-fmk (in which Z stands for benzyloxycarbonyl and fmk for fluoromethyl ketone) and microsequencing of the D4-GDI fragment revealed that this occurs at the caspase-3 cleavage site. Our results strongly suggest the differential regulation of the homologous GDP dissociation inhibitors Rho-GDI 1 and D4-GDI during drug-induced apoptosis by proteolysis mediated by caspase-3 but not by caspase-1. Owing to their crucial role as modulators of Rho GTPases, this might in turn have a significant impact on the mechanisms that induce the cytoskeletal and morphological changes in apoptotic cells.

Key words: epirubicin, mass spectrometry, microsequencing, taxol, two-dimensional electrophoresis.

INTRODUCTION

Apoptosis is an important process in a wide variety of biological systems, including physiological cell turnover, the immune system, embryonic development and hormone-dependent atrophy [1–4]. Interestingly, it has been shown that cytostatic agents such as taxotere [5], daunorubicin [6] and synthetic phospholipids [7] induce apoptosis, thereby providing some evidence for their mechanisms of action. The initiation of death signalling and the execution of the apoptotic programme after different death stimuli is mediated in most cases by a family of cysteine proteases called caspases [8]. Although death signalling in apoptosis is relatively well defined, especially for the death receptors located on the cell surface [9], the role of caspases as downstream effectors and the search for specific caspase substrates are continuing fields of research. In this context, the cleavage of the GDP dissociation inhibitor D4-GDI (Rho-GDI 2) has been demonstrated during CD95/Fas-induced and surface IgM-mediated apoptosis [10,11]; the relevance of this cleavage for the marked cytoskeletal changes that accompany apoptosis has been discussed.

Intensive studies have clarified the functions of small GTP-binding proteins, a protein superfamily which consists of the Ras, Rho, Arf, Rab and Ran families. It has been proposed that the Rho family of GTPases is involved in the regulation of integrin activity and in the organization of the actin cytoskeleton [12,13]. The activities of the different Rho proteins are regulated by at least three types of regulator protein: (1) GDP/GTP exchange proteins, (2) GTPase-activating proteins and (3) GDP dissociation inhibitors (GDIs) [14]. The last comprise a

family of proteins consisting of three members: Rho-GDI 1, D4-GDI (also named Rho-GDI 2 or Ly-GDI) and Rho-GDI 3. Rho-GDI 1 is expressed ubiquitously, whereas D4-GDI is expressed in haemopoietic tissues and Rho-GDI 3 in brain, lung, kidney, testis and pancreas [15].

The N-terminal cleavage of D4-GDI leads to the defective regulation of Rho-GTPase; truncated D4-GDI is unable to inhibit the dissociation of GDP from RhoA [16]. This might in turn have a negative impact on the organization of actin filaments.

However, the influence of cytostatic drugs on the status of the two members of the GDI family being expressed in lymphoid cells, Rho-GDI 1 and D4-GDI, has not yet been studied. In the present study, cleavage of GDI proteins after incubation of BJAB Burkitt-like lymphoma cells with taxol, a chemotherapeutic agent that perturbs microtubule dynamics [17], was investigated by Western blot analysis, high-resolution two-dimensional gel electrophoresis and subsequent microsequencing of the proteolytic cleavage site. Furthermore, the results were compared with the influence of epirubicin, another chemotherapeutic drug, which targets DNA and also induces G₂/M arrest. Our results demonstrate clearly that D4-GDI, but not Rho-GDI 1, is cleaved during taxol- and epirubicin-induced apoptosis, indicating that the proteolysis of D4-GDI is a highly specific feature of apoptosis, at least in haemopoietic cells.

Although caspase-1 has been shown to be involved mainly in inflammation (reviewed in [18]), previous studies suggested an additional role for caspase-1 in cell death after drug exposure [5,19]. Because D4-GDI contains caspase-1 and caspase-3 cleavage sites [10], we addressed the role of caspase-1 and the central effector caspase-3 in our experimental system. By using cell

Abbreviations used: BJAB, Burkitt-like lymphoma cell line; Caps, 3-(cyclohexylamino)propane-1-sulphonic acid; CBB, Coomassie Brilliant Blue; crmA, cytokine response modifier A; fmk, fluoromethyl ketone; GDI, GDP dissociation inhibitor; PARP, poly(ADP-ribose) polymerase; pNA, p-nitroanilide; Z, benzyloxycarbonyl.

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permeable peptide inhibitors we were able to show that caspase-1 is not involved in D4-GDI cleavage and apoptosis after treatment with taxol. Microsequencing of the resulting D4-GDI fragment led to the identification of the caspase-3 cleavage site. Thus cleavage of D4-GDI is independent from caspase-1 and occurs by a caspase-3-dependent mechanism.

EXPERIMENTAL

Materials

Polyclonal rabbit anti-(human caspase-3) (developed against human recombinant protein), anti-[poly(ADP-ribose) polymerase] (anti-PARP) (clone C2-10) and polyclonal rabbit anti-(human Rho-GDI/D4-GDI) (developed against full-length recombinant human Rho-GDI 1) antibodies were from Pharmingen (Hamburg, Germany). Secondary horseradish-peroxidase-conjugated anti-mouse antibodies and secondary horseradish-peroxidase-conjugated anti-rabbit antibodies were from Promega (Mannheim, Germany). Substrate for caspase-3-like activities, Ac-DEVD-pNA (in which pNA stands for *p*-nitroanilide), and substrate for caspase-1-like activities, Ac-YVAD-pNA, were from Calbiochem-Novabiochem GmbH (Bad Soden, Germany). Inhibitor for caspase-3-like activities, Z-DEVD-fmk (in which Z stands for benzyloxycarbonyl and fmk for fluoromethyl ketone), and caspase-1,4,5 inhibitor, Z-WEHD-fmk, were from Kamiya Biomedical Company (Seattle, WA, U.S.A.); they were dissolved in DMSO to give 20 mM stock solutions. According to the manufacturer, these inhibitors are synthesized as methyl esters to enhance cell permeability and facilitate the penetration of cell membranes; in cells the methyl groups are removed by endogenous enzymes. RNase A was from Roth (Karlsruhe, Germany). Epirubicin was purchased from Pharmacia Upjohn (Erlangen, Germany); taxol (paclitaxel) was from Bristol Arzneimittel GmbH (München, Germany).

Cell culture

BJAB cells transfected with control vector (mock transfection with pcDNA 3) and with pcDNA 3 crmA (cytokine response modifier A) [20] were kindly provided by Klaus Schulze-Osthoff (University of Tübingen, Tübingen, Germany) and were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 0.56 g/l L-glutamine, 100 000 i.u. penicillin and 0.1 g/l streptomycin. Media and culture reagents were from Life Technologies GmbH (Karlsruhe, Germany). Confluent cells were subcultured every 5 days by dilution of the cells to a concentration of 10^5 cells/ml.

Measurement of DNA fragmentation

Mock- and crmA-transfected BJAB cells were seeded at a density of 10^5 cells/ml and treated with different concentrations of the respective cytostatic drugs. Cells were harvested after 24, 48 and 72 h of incubation by centrifugation at 300 *g*. Cells were washed twice with cold PBS and fixed in PBS/2% (v/v) formaldehyde on ice for 30 min. After fixation, cells were incubated with ethanol/PBS (2:1, v/v) for 15 min, pelleted and resuspended in PBS containing 40 µg/ml RNase A. RNA was digested for 30 min at 37 °C, cells were pelleted again and finally resuspended in PBS containing 50 µg/ml propidium iodide. Nuclear DNA fragmentation was then quantified by flow-cytometric determination of hypodiploid DNA, as described [21]. Data were collected and analysed with a FACScan (Becton Dickinson, Heidelberg, Germany) equipped with CELLQuest software.

Data are given as percentages of hypoploidy sub- G_1 , which reflects the number of apoptotic cells.

Immunoblotting

After incubation with the respective cytostatic drugs, mock- and crmA-transfected BJAB cells were washed twice with PBS and lysed in buffer L [10 mM Tris/HCl (pH 7.5)/300 mM NaCl/1% Triton X-100/2 mM $MgCl_2$ /5 mM EDTA/1 µM pepstatin/1 µM leupeptin/0.1 mM PMSF]. Protein concentration was determined with the bicinchoninic acid assay [22] (Pierce, Rockford, IL, U.S.A.) and equal amounts of protein (usually 20 µg per lane) were separated by SDS/PAGE [23]; immunoblotting was performed essentially as described [24]. Membranes (Schleicher and Schuell, Dassel, Germany) were swollen in Caps buffer [10 mM Caps [3-(cyclohexylamino) propane-1-sulphonic acid] (pH 11)/10% (v/v) methanol] for several minutes and blotting was performed at 1 mA/cm² for 1 h in a transblot SD cell (Bio-Rad, München, Germany). The membrane was blocked for 1 h in blocking buffer [PBS/0.05% (v/v) Tween 20/3% (w/v) non-fat dried milk] and incubated with primary antibody in blocking buffer for 1 h. After the membrane had been washed three times in PBST [PBS/0.05% (v/v) Tween 20], secondary antibody was applied in PBST for 1 h. Finally, the membrane was washed in PBST again and bands were detected with the ECL[®] enhanced chemiluminescence system (Amersham Buchler, Braunschweig, Germany) in accordance with the manufacturer's protocol. Two-dimensional Western blots were prepared in the same way.

Two-dimensional gel electrophoresis

Cell pellets were spun down at 724 *g* (3000 rev./min) in an Eppendorf centrifuge and resuspended in 1 µl of buffer PE/mg of cells [PE buffer is 50 mM Tris/HCl (pH 7.1)/100 mM KCl/6 mM EDTA]. Ampholytes (pH 2–4) were added to a final concentration of 2% (v/v), sulphide bonds were reduced with 70 mM dithiothreitol and the mixture was supplemented with 1 mM PMSF and 1 µg/ml pepstatin. To achieve lysis and denaturing of proteins, urea was added to a final concentration of 9 M. DNA was separated by centrifugation at 70 000 *g* for 20 min at 20 °C. Aliquots (10 µl for 0.09 cm rod gels; 20–30 µl for 0.15 cm rod gels) of the samples were separated in the first dimension by using carrier ampholytes of pH 2–11 and 4% (w/v) gels 23 cm in length. After isoelectric focusing, proteins were separated by SDS/PAGE on 23 cm × 35 cm gels [15% (v/v) polyacrylamide] [25]. Staining was performed with Coomassie Brilliant Blue (CBB) G250 (0.15 cm rod gels) or silver staining as described by Blum et al. [26].

Identification of spots

Spots were identified by mass spectrometric methods [27] after tryptic digestion as described elsewhere [28]. The measurements were performed with a Q-ToF (Micromass, Manchester, U.K.) equipped with a nanoflow Z spray ion source. An aliquot of 1 µl was sufficient for one mass spectrum and five or six MS/MS experiments to obtain sequence information direct from the peptides in the mixture. Protein identification was possible for the faintest CBB-stained spots and not too weakly silver-stained spots. We used the sequence tag program, which combines partial manual spectrum interpretation of approx. three amino acids (sequence tag) with the residual mass N-terminal and C-terminal of the interpreted region and the peptide mass [29]. This information was then applied to searching in a non-redundant translated nucleotide database (<http://www.mann.embl>).

heidelberg.de/Services/PeptideSearch/FR_Peptide_Pattern-Form.html).

Preparation of cell extracts and induction of the cell-free apoptotic system

Cell extracts were prepared as described [30]. In brief, BJAB cells were washed twice with PBS, resuspended in buffer A [20 mM Hepes (pH 7.4)/10 mM KCl/2 mM MgCl₂/1 mM EDTA] and incubated on ice for 15 min. PMSF was added to give a final concentration of 0.1 mM. Cells were then disrupted by passage 15 times through a 21 G × 1½ needle (0.80 mm × 40 mm; Braun, Melsungen, Germany). The homogenates were cleared by centrifugation at 16000 *g* and 4 °C for 15 min. The centrifugation step was repeated and the clear supernatants were used for caspase activation *in vitro* or the measurement of caspase activities *in vitro*. For initiating caspase activation *in vitro*, 1 mM dithiothreitol, 1 µM horse heart cytochrome *c* (Sigma, München, Germany) and 1 mM dATP were added and the extracts were incubated at 30 °C for 5 min followed by incubation at 37 °C for different periods.

Measurement of caspase-3-like and caspase-1-like activity

After incubation with the respective cytostatic drugs, the mock- or crmA-transfected BJAB cells were washed twice with PBS and cell extracts were prepared as described above. After determination of the protein concentration with the bicinchoninic acid assay, caspase-3-like activity was measured as described [31], with some modifications. For this, 10 µl of extract, 90 µl of buffer B [50 mM Hepes (pH 7.4)/100 mM NaCl/1 mM EDTA/0.1% CHAPS/10% (w/v) sucrose/5 mM dithiothreitol] and 2 µl of colorimetric substrate (10 mM Ac-DEVD-pNA in DMSO) were mixed. Samples were incubated at 37 °C and A₄₀₅ was measured in an ELISA reader every 10 min. From the specific caspase-3 activities (units/mg of protein) the increase of caspase-3 activity in drug-treated cells over the respective controls was calculated.

Similarly, caspase-1-like activity was measured in BJAB cells by using Ac-YVAD-pNA as substrate.

RESULTS AND DISCUSSION

Induction of apoptosis, caspase-3 activation and cleavage of D4-GDI during drug-induced apoptosis

To examine D4-GDI cleavage after treatment with chemotherapeutic drugs, we first determined the concentration-dependent proapoptotic effects of taxol and epirubicin in mock-transfected BJAB cells and found that 0.12 µM taxol and 1.85 µM epirubicin efficiently induced DNA fragmentation (results not shown). Time-course experiments in mock-transfected BJAB cells revealed that DNA fragmentation was hardly observed after 24 h of incubation and increased significantly with time. After 72 h of incubation with 0.12 µM taxol or 1.85 µM epirubicin, the number of apoptotic cells reached 50–60% (Figure 1A). In a second set of experiments, we examined the cleavage of caspase substrates and the activation of caspase-3 by Western blot analyses with anti-(Rho-GDI) 1/D4-GDI, anti-caspase-3 and anti-PARP antibodies. In mock-transfected BJAB cells, cleavage of Rho-GDI 1/D4-GDI (Figure 1B) and cleavage of the typical death substrate PARP [32] (Figure 1E) were shown to be early events in drug-induced apoptosis occurring after 24 h of incubation and reaching a plateau after 48 h. This coincided with the activation of caspase-3 as shown by the appearance of the active 17 kDa subunit after 24, 48 and 72 h (Figure 1D).

Figure 1 Taxol and epirubicin time-dependently induce DNA fragmentation, cleavage of Rho-GDI 1/D4-GDI, activation of caspase-3 and cleavage of PARP

(A) Mock-transfected BJAB cells and BJAB/crmA cells were incubated for 24, 48 and 72 h with control medium (c1, c2 and c3 respectively), 0.12 µM taxol (t1, t2 and t3 respectively) or 1.85 µM epirubicin (e1, e2 and e3 respectively). DNA fragmentation was then determined as described in the Experimental section. Values are given as percentages of apoptosis. Results are representative of three separate experiments. (B, D, E) Extracts from mock-transfected BJAB cells that had been treated as described above were subjected to Western blot analyses with anti-(Rho-GDI) 1/D4-GDI (B), anti-(caspase-3) (D) and anti-PARP (E) antibodies, as described in the Experimental section. Lanes M, molecular mass marker. (C) In addition, BJAB/crmA cells were treated in the same way and Western blot analysis was performed with anti-(Rho-GDI) 1/D4-GDI antibody. Results shown are representative of three separate experiments. Lane M, Molecular mass marker. kD, kDa.

Measurement of caspase-3 activity after 48 h of incubation showed 16-fold and 23-fold increases in caspase-3-like activity in taxol- and epirubicin-treated mock-transfected BJAB cells respectively. In these experiments, no activation of caspase-1-like activity was detected (results not shown). Furthermore, we investigated caspase-dependent cleavage of Rho-GDI 1/D4-GDI by using BJAB cells transfected with the cowpox virus gene crmA, which encodes a protease inhibitor of the serpin family. crmA has been described as a potent inhibitor of Fas- and tumour-necrosis-factor-induced apoptosis [20], interfering preferentially with caspase-1 and caspase-8 (reviewed in [33]). As shown in Figure 1(A), crmA inhibited taxol- and epirubicin-induced apoptosis but the inhibition was not complete. The same was true for the cleavage of Rho-GDI 1/D4-GDI which was decreased in crmA-transfected BJAB cells after 24 h of incubation (Figure 1C) in comparison with mock-transfected BJAB cells (Figure 1B). However, significant cleavage of Rho-GDI

Figure 2 Time-dependent cleavage of PARP and Rho-GDI 1/D4-GDI after activation of caspases *in vitro* and localization of Rho-GDI 1, D4-GDI and D4-GDI fragment on a two-dimensional Western blot

(A, B) Extracts of mock-transfected BJAB cells were incubated with 10 μ M cytochrome *c* and 1 mM dATP for different durations as indicated. The reaction was then stopped by the addition of SDS sample buffer, and Western blot analyses were performed with anti-PARP (A) and anti-(Rho-GDI 1/D4-GDI) (B) antibodies as described in the Experimental section. The positions of molecular mass markers are indicated (in kDa) at the right. (C) Additionally, the cytochrome *c*/dATP-treated extract of mock-transfected BJAB cells was separated by high-resolution two-dimensional gel electrophoresis, and Western blot analysis was performed with anti-(Rho-GDI 1/D4-GDI) as described in the Experimental section. Arrows indicate the positions of spots 1, 2 and 2* (see also Figure 4). The experiments were repeated and yielded similar results.

1/D4-GDI was observed after 48 and 72 h in drug-treated crmA-transfected cells (Figure 1C). Similarly, caspase-3 activation as well as PARP cleavage after 24 h were decreased in crmA-transfected cultures in comparison with mock-transfected BJAB cells but reached the same level as mock-transfected BJAB cells after 72 h (results not shown). Thus the inhibition of caspase-1 by crmA does not prevent the cleavage of D4-GDI, indicating that caspase-1 is not essential for D4-GDI cleavage.

Our results are consistent with earlier reports that demonstrated a rapid, caspase-3-dependent, cleavage of D4-GDI after treatment of lymphoid and myeloid cells with death-inducing stimuli such as CD95/Fas and anti-IgM [10,11]. However, cleavage of Rho-GDI 1/D4-GDI in our experimental system was not complete after 72 h of incubation with taxol or epirubicin (Figure 1B). To exclude the possibility that this was simply due to the presence of non-apoptotic cells in the samples used for the Western blot analyses, we established a cell-free system based on the activation of cytosol with cytochrome *c* and dATP, as described by Liu et al. [34]. In this system *in vitro*, a proteolytic cascade is initiated that leads to the rapid activation of caspase-3 [35]. In contrast with PARP, which was completely cleaved 25 min after the addition of 10 μ M cytochrome *c* and 1 mM dATP to the cytosolic extract of mock-transfected BJAB cells (Figure 2A), the band corresponding to Rho-GDI 1/D4-GDI did not disappear, even after 185 min (Figure 2B).

Detecting specific D4-GDI cleavage by two-dimensional gel electrophoresis and MS

Keeping in mind that the antiserum employed for Western blot analysis detects both Rho-GDI 1 and D4-GDI, we decided to study the cleavage of the two related proteins by high-resolution

	1				50
Rho-GDI 1	MAEQEPTAEQ	LAQIAAENE	DEHSVNYKPP	AQKSIQEIQZ	LDKDDESLRK
D4-GDI	MTEKAPEPHV	EDDDDELD	KLNKPPPPQK	SLKELQEMDK	DDESLIKYKK
D4-GDI*	S	S	KLNKPPPPQK	SLKELQEMDK	DDESLIKYKK
	51				100
Rho-GDI 1	YKEALLGRVA	VSADPNPNV	VVTGLTLVCS	SAPGPLELDL	TGDLSEFKK
D4-GDI	TLLGDGPPVT	DPKAPNVVT	RLTLVCSAP	GPITMDLTGD	LEALKKETIV
D4-GDI*	TLLGDGPPVT	DPKAPNVVT	RLTLVCSAP	GPITMDLTGD	LEALKKETIV
	101				150
Rho-GDI 1	SEVLKEGVEY	RIKISFRNR	EIVSGMKYIQ	HTYRKGVKID	KTDYMGVSYG
D4-GDI	LKEGSEYRVK	IHFKNRDIR	SGLKTVQHTY	RTGVKVDKAT	FMVGSYGPRP
D4-GDI*	LKEGSEYRVK	IHFKNRDIR	SGLKTVQHTY	RTGVKVDKAT	FMVGSYGPRP
	151				200
Rho-GDI 1	PRAEYEFLT	FVEEAPKGL	ARGSYSIKSR	FTDDDKTDHL	SWEWNITIKK
D4-GDI	EYEYFLTPVE	EAPKGLARG	TYHNKSFSTD	DDKQDHLSE	WNLSIKKEWT
D4-GDI*	EYEYFLTPVE	EAPKGLARG	TYHNKSFSTD	DDKQDHLSE	WNLSIKKEWT
	201				
Rho-GDI 1	DWKD				
D4-GDI	E				
D4-GDI*	E				

Figure 3 Characterization of spots 1, 2 and 2* by MS

Spots 1, 2 and 2* were cut from a CBB-stained two-dimensional electrophoresis gel. The proteins were digested and peptide masses were detected in the MS spectra (bold italics). Peptide sequences obtained by MS/MS are printed in bold. Aligned sequences of Rho-GDI 1 (spot 1), D4-GDI (spot 2) and fragment of D4-GDI (spot 2*) are shown.

two-dimensional gel electrophoresis. First, the spots representing the two GDI proteins and putative fragments were localized by Western blot analysis of an cell extract activated *in vitro* and separated by two-dimensional gel electrophoresis. With this technique, the two bands detected after one-dimensional separation by SDS/PAGE (Figure 2B) were separated into three spots: 1, 2 and 2* (Figure 2C). The identities of the indicated spots were verified by MS: spot 1 was Rho-GDI 1 and spot 2 was D4-GDI, as was spot 2* (Figure 3). However, the apparent molecular mass of 23 kDa and the pI of approx. 6.4 deduced from the position of spot 2* on the two-dimensional gel matched the theoretically predicted values for the corresponding fragment resulting from the cleavage of D4-GDI at Asp¹⁹, as described by Na et al. [10]. Thus, spot 2* was undoubtedly a fragment of D4-GDI.

To investigate the specificity of Rho-GDI 1/D4-GDI cleavage, whole-cell lysates from mock-transfected BJAB cells that had been treated with chemotherapeutic drugs were separated by high-resolution two-dimensional gel electrophoresis. Then the intensities of the corresponding spots after silver staining of the proteins were compared with the respective controls. As shown in Figure 4(A) (upper panels), Rho-GDI 1 (spot 1) did not show any decrease in intensity after 72 h of incubation with 0.12 μ M taxol, whereas D4-GDI (spot 2) disappeared in the same lysates. Additional time-course experiments revealed that the intensity of D4-GDI was already declining after 24 h of incubation (results not shown). In contrast, the protein levels of Rho-GDI 1 showed only marginal fluctuations after 24, 48 and 72 h of incubation, indicating that this protein was resistant to cleavage by different caspases or even resistant to proteolytic cleavage in general. In contrast, the D4-GDI fragment (spot 2*) was not visible in control cells and appeared only on the induction of apoptosis (Figure 4A, lower panels). Similar results were obtained when the cells were treated with 1.85 μ M epirubicin (results not shown). We also analysed the specificity of Rho-GDI 1/D4-GDI cleavage in the cell-free dATP/cytochrome *c*-activated system [34]. The results were virtually the same as described for the lysates of drug-treated cells: whereas the spot intensity of D4-GDI in the activated extract decreased in comparison with the control extract, Rho-GDI 1 remained unchanged (Figure 4B, upper panels). Furthermore, cleavage of D4-GDI in the extract activ-

Figure 4 Specific cleavage of D4-GDI in apoptotic cells and in cell extracts activated *in vitro*

(A) Mock-transfected BJAB cells were incubated for 72 h with 0.12 μ M taxol (apoptotic cells) or no additional supplements (control cells). The cell extracts were then separated by high-resolution two-dimensional gel electrophoresis and proteins were stained with silver as described in the Experimental section. The upper panels show the parts of two-dimensional gels in which spots 1 and 2 migrated; the lower panels show the parts of two-dimensional gels that included spot 2* (see also Figure 2C). Assigned spots were cut from a CBB-stained two-dimensional gel that was run in parallel. The proteins were digested and identified by MS. Spot 1, Rho-GDI 1; spot 2, D4-GDI; spot 2*, fragment of D4-GDI. (B) Extracts of mock-transfected BJAB cells were incubated for 3 h with 10 μ M cytochrome *c* and 1 mM dATP (*in vitro* activated) or no additional supplements (control). The cell extracts were then separated by high-resolution two-dimensional gel electrophoresis and proteins were stained with silver as described in the Experimental section. The upper panels show the parts of two-dimensional gels in which spots 1 and 2 migrated; the lower panels show the parts of a two-dimensional gel that included spot 2* (see also Figure 2C). Spot assignments are as in (A). The experiments were repeated and yielded similar results.

ated *in vitro* was accompanied by the appearance of the corresponding fragment (Figure 4B, lower panels). These results clearly confirmed the specific cleavage of D4-GDI but not Rho-GDI 1 during drug-induced apoptosis, thereby providing further evidence that other potential cleavage sites that are present in Rho-GDI 1 (e.g. LELD⁸⁹L or DKTD¹⁴³Y) are not accessible. The experiments *in vitro* with cell-free extracts indicate that cleavage of D4-GDI also occurs downstream of the cytochrome *c*- and dATP-dependent formation of the Apaf-1/caspase-9 complex [36] and is not restricted to death-receptor-mediated apoptosis. The time course of D4-GDI cleavage *in vitro* (see Figure 2B) closely resembled the time courses of other typical death substrates such as Fodrin, U1sn-RNP and PARP [35].

D4-GDI is proteolysed by caspase-3

Triggering of the death cascade by cytotoxic drugs has recently been shown to lead to CD95/Fas-independent caspase activation [37]. In this context, an involvement of caspase-3 in taxol-induced cell death has been demonstrated in different cell types [38,39] but the role of caspase-1 in drug-mediated cytotoxicity is still a matter of debate. For example, in mouse fibroblast L929 cells a time-dependent increase of interleukin-1 β -converting enzyme-like activity has been reported after treatment with taxotere [5]. Interestingly, D4-GDI is a substrate for both caspase-1 [16] and caspase-3 [10] and a detailed analysis of D4-GDI cleavage should reveal the role of these proteolytic enzymes

Figure 5 Taxol-induced cleavage of D4-GDI is inhibited by a cell-permeable inhibitor of caspase-3-like activity

Mock-transfected BJAB cells were preincubated for 2 h with different concentrations of Z-DEVD-fmk, Z-WEHD-fmk or diluent alone as indicated. The cells were then treated for 24 h with 0.12 μ M taxol. Controls (C) were incubated in the absence of taxol. Cell extracts were prepared and subjected to Western blot analyses with anti-(Rho-GDI 1/D4-GDI) as described in the Experimental section. The experiment was repeated and yielded similar results.

in drug-induced apoptosis. First, we analysed the cleavage site of the D4-GDI fragment that appeared after the treatment of BJAB cells with taxol and also with epirubicin. However, N-terminal sequencing needs approx. 10 pmol of peptide; because it is very difficult to get enough protein from apoptotic cells to do HPLC and N-terminal Edman sequencing we decided to use microsequencing by nanospray MS. This method allows sequencing with femtomole amounts of a peptide mixture and enabled us to distinguish between the caspase-1 and caspase-3 cleavage sites of the D4-GDI fragment (spot 2*) in cellular extracts from apoptotic cells. Microsequencing of tryptic peptides of spot 2* by MS/MS led to the identification of the peptide E³⁴LQEMDKDDESLIK⁴⁷ (Figure 3). Because the cleavage site of caspase-3 is represented by DELD¹⁹S and the cleavage site of caspase-1 by LLGD⁵⁵G [10], the detection of this peptide, located between Lys³³ and Tyr⁴⁸ of spot 2*, was consistent only with the caspase-3-mediated proteolysis of D4-GDI. This was confirmed by inhibitor experiments with cell-permeable peptide inhibitors. Caspases can be divided into different groups on the basis of their substrate specificities (reviewed in [40]). By using a combinatorial approach, specificities of members of the caspase family were defined by Thornberry et al. [41] as follows: caspase-3-like activities display the subsite preference DEXD and caspase-1-like activities (W/L)EHD. We therefore used in our experimental system Z-DEVD-fmk or Z-WEHD-fmk, which irreversibly inhibit caspase-3-like and caspase-1-like activities respectively. As shown in Figure 5, 10 μ M Z-DEVD-fmk blocked the taxol-induced cleavage of D4-GDI, whereas Z-WEHD-fmk had little or no effect.

Novel aspects of D4-GDI in comparison with Rho-GDI 1 cleavage in drug-induced apoptosis

As already outlined above, D4-GDI cleavage has been shown to occur after receptor-mediated apoptosis [10,11]. By mixing recombinant proteins in an assay *in vitro*, it has been demonstrated that only D4-GDI, and not Rho-GDI 1, represents a substrate for caspase-mediated cleavage [16]. In our study we used neither purified caspases nor recombinant substrates. In contrast, we analysed cleavage *in vivo* and employed whole-cell lysates without further purification in combination with the described analytical methods and the application of caspase inhibitors. We were thus able to demonstrate for the first time that (1) D4-GDI is cleaved during drug-induced apoptosis, (2) D4-GDI cleavage is specific because Rho-GDI 1 is not cleaved

in vivo (either by caspases or by other proteases) when BJAB cells undergo apoptosis, (3) D4-GDI is cleaved *in vivo* at the caspase-3 cleavage site after treatment of BJAB cells with drugs, and (4) caspase-1 does not have a role in the taxol-induced cleavage of D4-GDI and the subsequent induction of apoptosis in BJAB cells. The last is of interest for the understanding of taxol-induced cell death because caspase-1 has previously been implicated in the induction of apoptosis by this kind of compound.

Taken together, the differential, caspase-3-mediated cleavage of D4-GDI in comparison with Rho-GDI 1 during drug-induced apoptosis indicates that, despite their high degree of similarity, these proteins display different properties. In this context it is interesting to note that the differential phosphorylation and Rho-GTPase selectivity of D4-GDI compared with Rho-GDI 1 have been shown after the stimulation of U937 cells with phorbol ester [42]. This might also be related to the differential effect of a D4-GDI knock-out in mice on apoptosis regulation. Only the apoptosis induced by IL-2 withdrawal, but not dexamethasone- and T-cell-receptor-induced apoptosis, was affected in lymphocytes from D4-GDI-deficient mice [43]. Thus the relevance of D4-GDI cleavage to the absence of Rho-GDI 1 cleavage for different apoptotic pathways might be an interesting subject for further investigation.

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