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Prolactin blocks glucocorticoid induced cell death by inhibiting the disruption of the mitochondrial membrane

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Abstract

Prolactin (PRL) has been reported to inhibit dexamethasone (Dex) induced cell death. Nevertheless, the mechanism through which PRL exerts its protective effect is still not unravelled. Here, we analyse the effect of PRL at different stages of the glucocorticoid (GC) apoptotic pathway in PRL dependent cells (Nb_2 cells). PRL blocks completely the GC induced loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$) and consequently phosphatidylserine (PS) exposure and loss of DNA content. Although PRL promotes an upregulation of the bcl-2 expression, simultaneous addition of PRL to GC fails to maintain even the normal levels of this anti-apoptotic protein. This finding excludes a critical role for bcl-2 in the PRL protective effect against GC. GC induced $\Delta\Psi_m$ disruption can be inhibited by the ICE-like inhibitor zVAD-fmk but not by ICE inhibitor tetrapeptide acetyl-Tyr-Val-Ala-Asp.chloromethylketone (YVAD-cmk) nor by caspase-3 inhibitor zDEVD. It can be speculated that PRL blocks $\Delta\Psi_m$ disruption by inhibiting an unknown caspase activated by GC.

Keywords: Apoptosis; Prolactin; Dexamethasone; Mitochondrial transmembrane potential; bcl-2; ICE-like proteases

1. Introduction

Glucocorticoid (GC) hormones have a broad spectrum for medical applications. They are known to possess suppressive effects on the immune system and are consequently used as anti-inflammatory, immunosuppressive or cytostatic drugs. Moreover, GCs can activate the suicide program in lymphocytes and thymocytes. To date neither the way of signal transduction of the GC–GC-receptor pathway nor the mechanism of action of GC mediated apoptosis are fully understood.

In Nb_2 cells, a rat pre-T lymphoma cell line, the synthetic GC, dexamethasone (Dex) is capable to induce programmed cell death, leading to typical hallmarks of apoptosis such as DNA fragmentation [1]. The antagonist RU486 can directly inhibit GC induced apoptosis which indicates a direct action through the GC receptor [2]. In the cytosol the GC receptor is associated with proteins such as heat shock protein 90 (hsp90). As soon as the GC binding occurs, the GC-receptor dissociates from hsp90 and translocates the GC–GC receptor complex from the cytosol to the nucleus [3]. GCs need protein synthesis, indicated by the fact that the protein synthesis inhibitor cycloheximide can prevent steroid induced cell death in T-lymphocytes [4]. The GC–GC-receptor complex acts as a translation regulatory factor and leads to the expression of specific proteins that presumably induce apoptosis. The ligation between GC and GC-receptor results in the induction of T-cell apoptosis. It can be speculated that this event is mediated through an alternative pathway, interacting with transcription factors such as activator protein-1 (AP-1) [5]. AP-1 (*Jun*/*Fos*) which is involved in cell proliferation is a dimeric protein complex formed by members of the *Jun* (*c-jun*, *JunB*, *JunD*) and *Fos* (*cFos*,

Abbreviations: C_2, ceramide; Dex, dexamethasone; GC, glucocorticoid; Nb_2 cell line, prolactin dependent cell line; PI, propidium iodine; PRL, prolactin; PS, phosphatidylserine; YVAD, tetrapeptide acetyl-Tyr-Val-Ala-Asp.chloromethylketone; zDEVD.fmk, Z-Asp-Glu-Val-Asp-CH_2F; z-VAD.fmk, N-benzyloxycarbonyl-Val-Ala-Asp.fluoromethylketone.

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FosB, *Fra-1*, *Fra-2*) protein family [6]. Whereas members of the *Jun* family can form homo- and heterodimers, *Fos* members can only form heterodimers with *Jun* members. There is evidence that GCs regulates *c-jun* gene expression in programmed cell death [7]. In addition, Dex causes a downregulation of c-myc expression in GC evoked apoptosis [8].

GCs lead to a selective increase of calcium-independent protein kinases C (PKC δ, ε, η (L), θ, ξ, λ) in immature thymocytes but not in mature T-cells. PKC inhibitors can prevent GC induced apoptosis [9]. Dex causes the breakdown of the inner mitochondrial transmembrane potential $\Delta\Psi_m$ [10] which is implicated to be an irreversible phenomenon. Consecutively, GCs activate enzymatically pro-CPP32 to its subunits and lead to the cleavage of 116 kD PARP (poly (ADP) polymerase) into its 85 kD and 25 kD fragments [11].

Prolactin (PRL) seems to protect the mammary gland in undergoing apoptosis [12,13]. Furthermore, PRL prevents GC induced apoptosis in lymphoid cells [1]. Buckley and co-workers showed that PRL causes an increase in the apoptosis regulatory gene expression of bcl-2 mRNA [15]. Bcl-2 is expressed in the outer mitochondrial membrane, the nuclear membrane and the endoplasmatic reticulum [15,16] and in general prevents apoptosis by inhibiting mitochondrial alterations such as large amplitude swelling and permeability transition [17]. Up to now the role of PRL in the process of Dex induced apoptosis remains elusive. Here, we obtain evidence that PRL inhibits the loss of the transmembrane potential that is caused by Dex. However, bcl-2 does not seem to play a critical role in this such process. It can be speculated that PRL prevents the transmembrane disruption by inhibiting an unknown caspase induced by GCs.

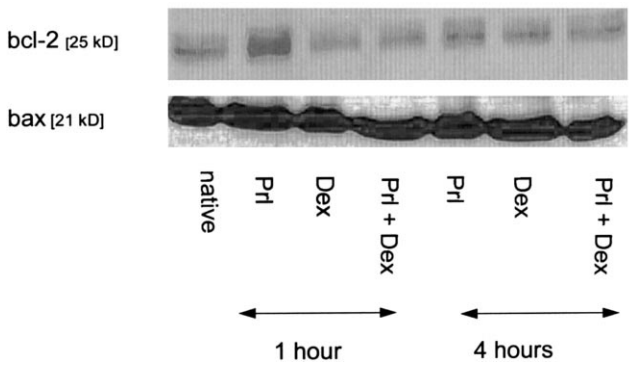


Fig. 1. Western blot of bcl-2 and bax-expression. Cells were stimulated for 1 and 4 h with Dex (10 μM) and/or oPRL (100 ng/ml).

2. Methods

2.1. Cells and culture condition

The PRL-dependent rat pre-T lymphoma cell line was obtained from Dr PW Gout (Vancouver British Columbia, Canada). Nb_2 cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated foetal calf serum (FCS), 10% horse serum (Sigma), L-glutamine (Sigma), Hepes (ICN) and antibiotics (Sigma). β-Mercaptoethanol (50 μM, Sigma Chemicals Co) was added to the culture medium. Before stimulation cells were kept in starvation medium (without FCS) for at least 4 h to synchronise the cells into G_0/G_1 phase. To induce apoptosis different strategies were used: cells were stimulated with Dex (10 μM Soludecadron®, MDS) or cultured in the presence of ceramide (C_2, 50 μM, Sigma Chemical Co), etoposide (1 μM Sigma) or staurosporine (1 μM, Sigma), cycloheximide (1 μg/ml, Sigma) and/or ovine PRL (oPRL, 100 ng/ml). zVAD.fmk (100 μM, *N*-benzyloxycarbonyl-Val-AlaAsp.fluoromethylketone, Bachem, Bubendorf, Switzerland) as an inhibitor for most mammalian IL-1β converting enzyme (ICE)-like/ced-3 proteases (caspase), zDEVD.fmk (100 μM, Z-Asp-Glu-Val-Asp.fluoromethylketone, Enzyme Systems Products) a more selective inhibitor for caspase-3 (CPP32, Yama, Apopain) family proteases (caspase-3,-6,-7), and YVAD.cmk (100 μM; Bachem Switzerland, tetrapeptide acetyl-Tyr-Val-Ala-Asp.chloromethylketone) as a blocker of ICE were used as specific inhibitors of apoptosis.

2.2. Western blot analysis

Stationary Nb_2 cells were stimulated at various time points with oPRL (100 ng/ml) and/or Dex 10 μM. The cells were centrifuged and resuspended in lysis buffer containing 10 mM Tris–HCl (pH 7.4), 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride (PMSF) and 25 μl/ml each of leupeptin, pepstatin and aprotinin. The lysates were centrifuged for 10 min at $14000 \times g$ at 4°C. Total protein content was determined using the Bradford reagent (Bio-Rad, Richmond, USA). Lysates were fractionated by SDS-PAGE using 7.5–12.5% gels (100 μg/lane) and electrophoretically transferred to nitro-cellulose blotting membrane (Biorad). Membranes were blocked for 2 h in blocking buffer and incubated overnight at 4°C in antibody buffer with anti-rabbit bax (1:1000) or anti-rabbit bcl-2 (1:500) antibodies distributed by Santa Cruz. PARP (1:10 000) anti-mouse monoclonal antibody was used (Pharmingen). Proteins were visualised by chemiluminecence detection using secondary anti-

[%]	medium	PRL [100 ng/ml]
medium	4.4	1.6
Dex [10 μM]	26.3	2.8
Ceramide [50 μM]	13.3	2.6

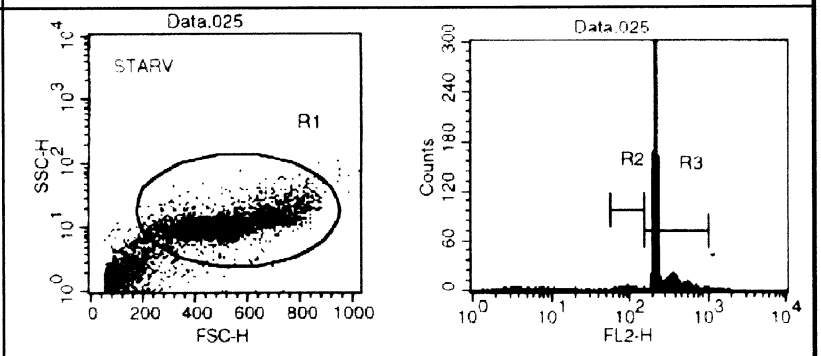
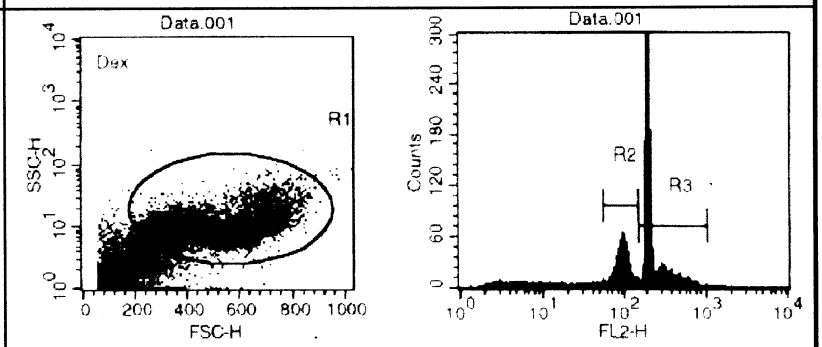
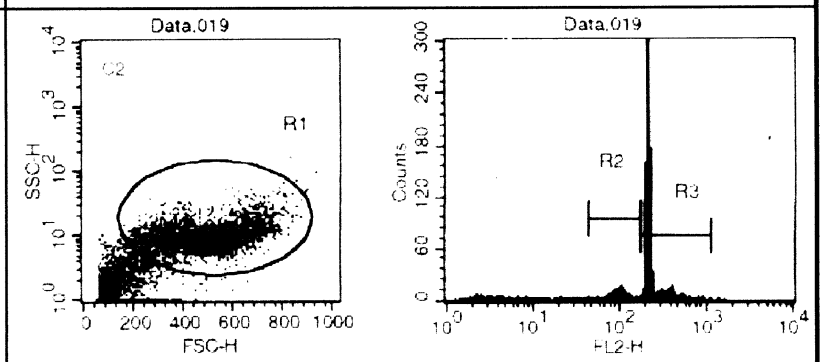
[%]	starv
Medium	
Dex [10 μM]	
Ceramide [50 μM]	

Fig. 2. Effects of PRL on pro-apoptotic substrates such as Dex and C_2. PRL inhibits C_2 and Dex induced cell death, cells were stimulated for 18 h and analysed by cell cycle analysis.

bodies coupled to alkaline phosphatase followed by exposure to X-ray films for 1–10 min.

2.3. Cytofluorometric analysis and assessment of hypoploidy

Loss of membrane integrity was determined by the vital dye propidium iodine (PI). The frequency of cells having lost part of their chromosomal DNA (subdipoid cells) was determined by PI staining of ethanol-permeabilised cells [18].

An FITC-annexin V conjugate (1:400 dilution, 1 μg/ml 15 min at 4°C, 525 nm, Brand Application, Maastricht, The Netherlands) with high affinity for phosphatidylserine (PS) was used for the assessment of aberrant PS exposure. Labelling with FITC-annexin V was performed after removal of FCS by washing cells twice in HEPES buffer (10 nM HEPES NaOH (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, and 1.8 mM $CaCl_2$).

2.4. Cytofluorometric analysis of mitochondrial parameters

3,3,dihexyloxacarbocyanine iodide ($DiOC_6$) was used as a cationic lipophilic fluorochrome (Molecular Probes) to measure $\Delta\Psi_m$. Cells were incubated at 37°C for 15 min in the presence of $DiOC_6$ (40 nM; 525 nm), followed by immediate analysis of fluorochrome incorporation in an Epics Profile II cytofluorometer (Coulter Corp., Miami, FL).

Enzymatic activation of CPP32 was detected by adding 1 μM Ac-DEVD-amino-4.methylcoumarin (30 min, 37°C). ZVAD.fmk (100 μM) was added in another experiment.

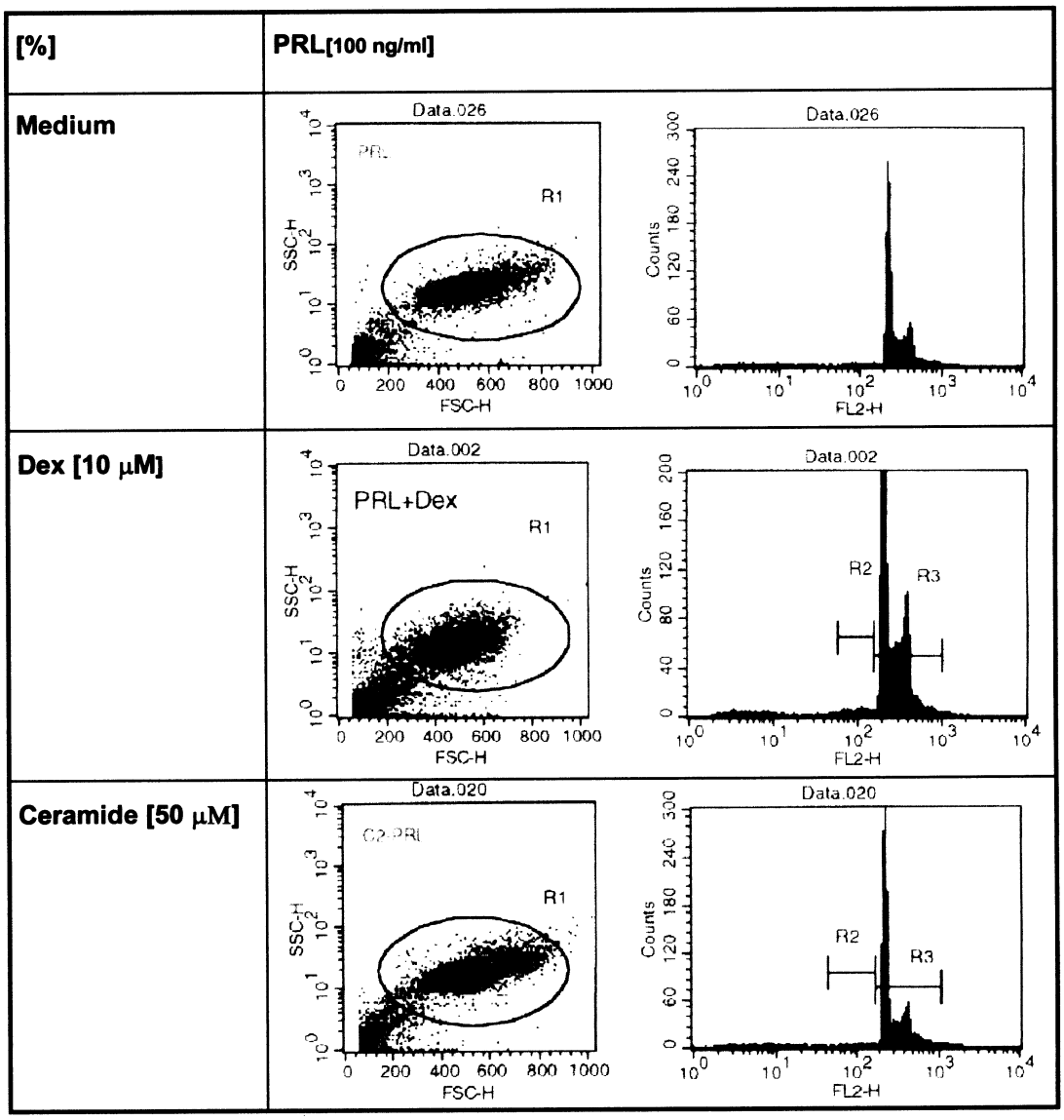


Fig. 2. (*Continued*)

[%]	medium	PRL [100 ng/ml]	CHX [1 ug/ml]	YVAD [100 μM]	zVAD [100 μM]	zDEVD [100 μM]
medium	19	14	18	14	22	15
ETP [1 μM]	46	55	24	38	46	48
STS [1 μM]	56	60	46	50	63	53
Dex [10 μM]	46	5.9	17	44	22	59

Fig. 3. Cell cycle analysis of various apoptosis inducers such as etoposide, staurosporine, Dex and cycloheximide (CHX). Effects of CHX and protease inhibitors (YVAD.cmk, zVAD.fmk, zDEVD.fmk) on different apoptotic signalling in Nb_2 cells. Results are representative of three different independent experiments. Cells were stimulated for 20 h.

3. Results

3.1. PRL does not inhibit bcl-2 downregulation mediated by Dex

PRL induces a time dependent upregulation of bcl-2 expression in Nb_2 cells (Fig. 1). Maximal increase of bcl-2 protein expression occurs after 1–2 h of stimulation whereas Dex leads to a diminution of bcl-2 expression shown by western blot analysis. These results suggest PRL inhibits apoptosis independently of bcl-2 upregulation. PRL however is not able to inhibit the negative effect of Dex via bcl-2. Bax expression remains unaltered by stimulation with hormones such as Dex, PRL or PRL + Dex.

In order to analyse the mechanism by which PRL inhibits the effect of GC apoptosis we studied various inducers of cell death. Nb_2 cells were stimulated in the presence of pro-apoptotic substrates such as: etoposide, an inactivator of DNA-topoisomerase and a reconstitutor of DNA; staurosporine, an inhibitor of PKC and C_2, a second messenger in the induction of apoptosis (Fig. 2). PRL sufficiently prevents C_2 and Dex induced cell death. Cycloheximide (CHX) being used as a protein synthesis inhibitor is capable to counteract Dex induced apoptosis in our cell model (Fig. 3). Prolactin neither uses protein kinase c nor DNA-topoisomerase for its signal transduction in the apoptotic process.

3.2. $\Delta\Psi_m$ is preserved by PRL treatment

Cells that are undergoing programmed cell death exhibit a fall in $\Delta\Psi_m$. $DiOC_6$ as a cationic lipophilic fluorochrome can be used as a dye that correlates with the mitochrondrial membrane potential. We demonstrate that PRL prevents the disruption of the mitochondrial membrane potential (Fig. 4). In conclusion, it blocks the induction of the apoptotic process by inhibiting the point of no return, the fall of the $\Delta\Psi_m$.

3.3. PRL inhibits the exposure of PS on the outer membrane that is being induced by Dex

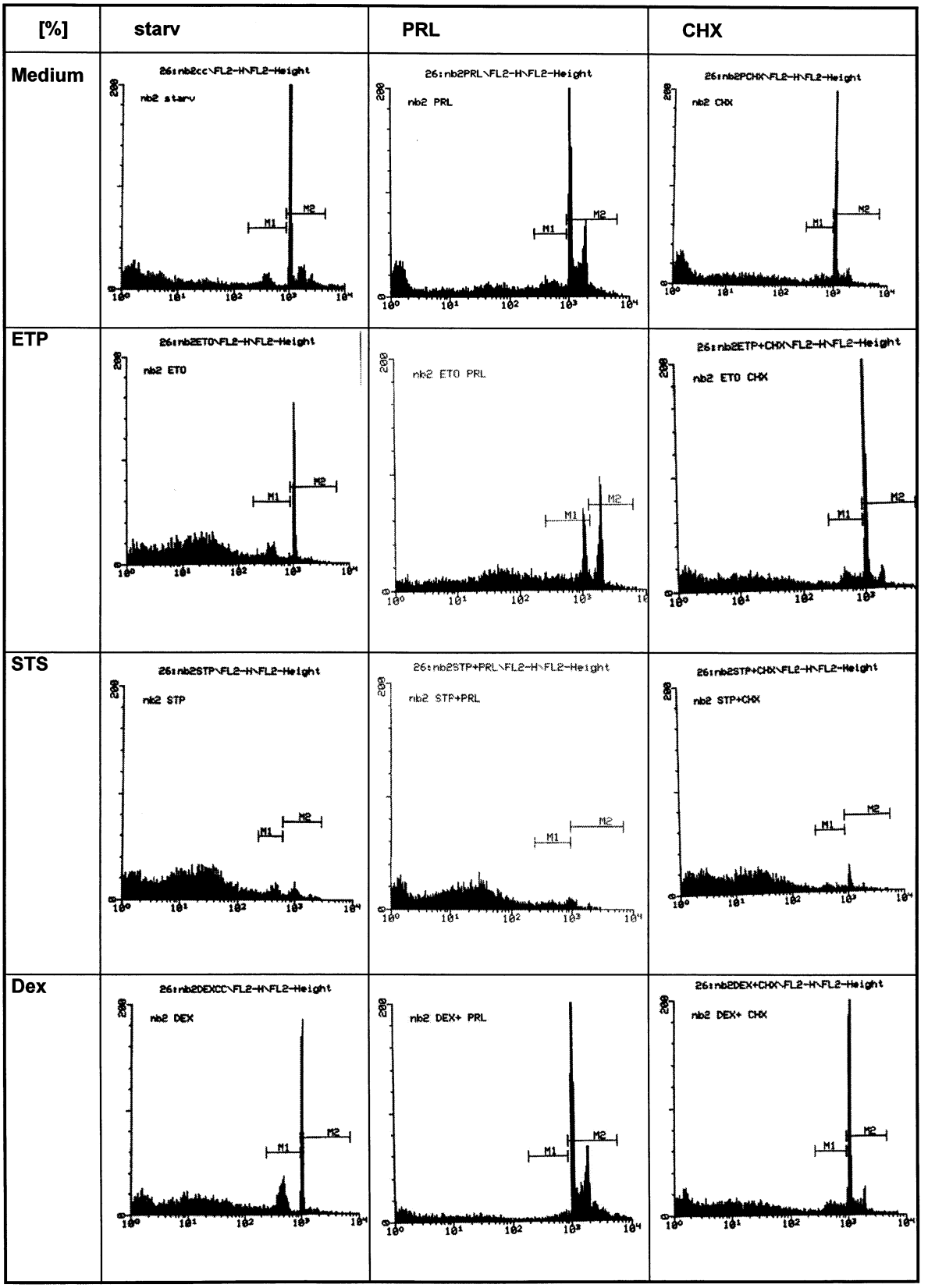
Before cells loose their viability, they expose PS residues on the outer plasma membrane leaflet in contrast to normal, quiescent cells. Comparable with other cell models Dex leads to the exposure of PS as a typical feature of the apoptotic process in Nb_2 cells (Fig. 5). The addition of PRL blocks the exposure and flipping out of PS residues on the outer cell membrane leaflet, indicating that PRL acts as an early common event in the apoptotic cascade. The fragmentation and loss of DNA (hypoploidy) by stimulation with Dex becomes apparent after 8–12 h per FACS analysis by stimulation with Dex. PRL is able to prevent this loss of DNA.

3.4. PARP cleavage is not specific for Dex induced apoptosis.

Dex is reported to lead to the cleavage of 116 kD polymerase PARP (poly (ADP) polymerase) into its 85 kD fragment [11]. Analysis of PARP by western blot analysis reveals that PARP is already cleaved in proliferating Nb_2 cells (Fig. 6). Consecutively, we measured DEVDase that is metabolised by CPP32 (Fig. 7). DEVDase activity is upregulated in normally growing Nb_2 cells. This effect can not be significantly augmented under Dex treatment nor be blocked by the co-stimulation of PRL. Moreover, cells proliferate even in the presence of activated CPP32. This indicates that CPP32 activation occurs independently of Dex induced cell death. These results confirm our data obtained by western blot analysis.

3.5. PRL possibly blocks the disruption of the mitochondrial membrane by inhibiting a caspase induced by Dex.

Dex leads to the loss of the mitochondrial potential. zVAD.fmk, a specific inhibitor of ICE-like protease could inhibit Dex induced cell death in Nb_2 cells whereas the ICE inhibitor YVAD.cmk and the CPP32 proteases inhibitor zDEVD.fmk fail to interrupt the



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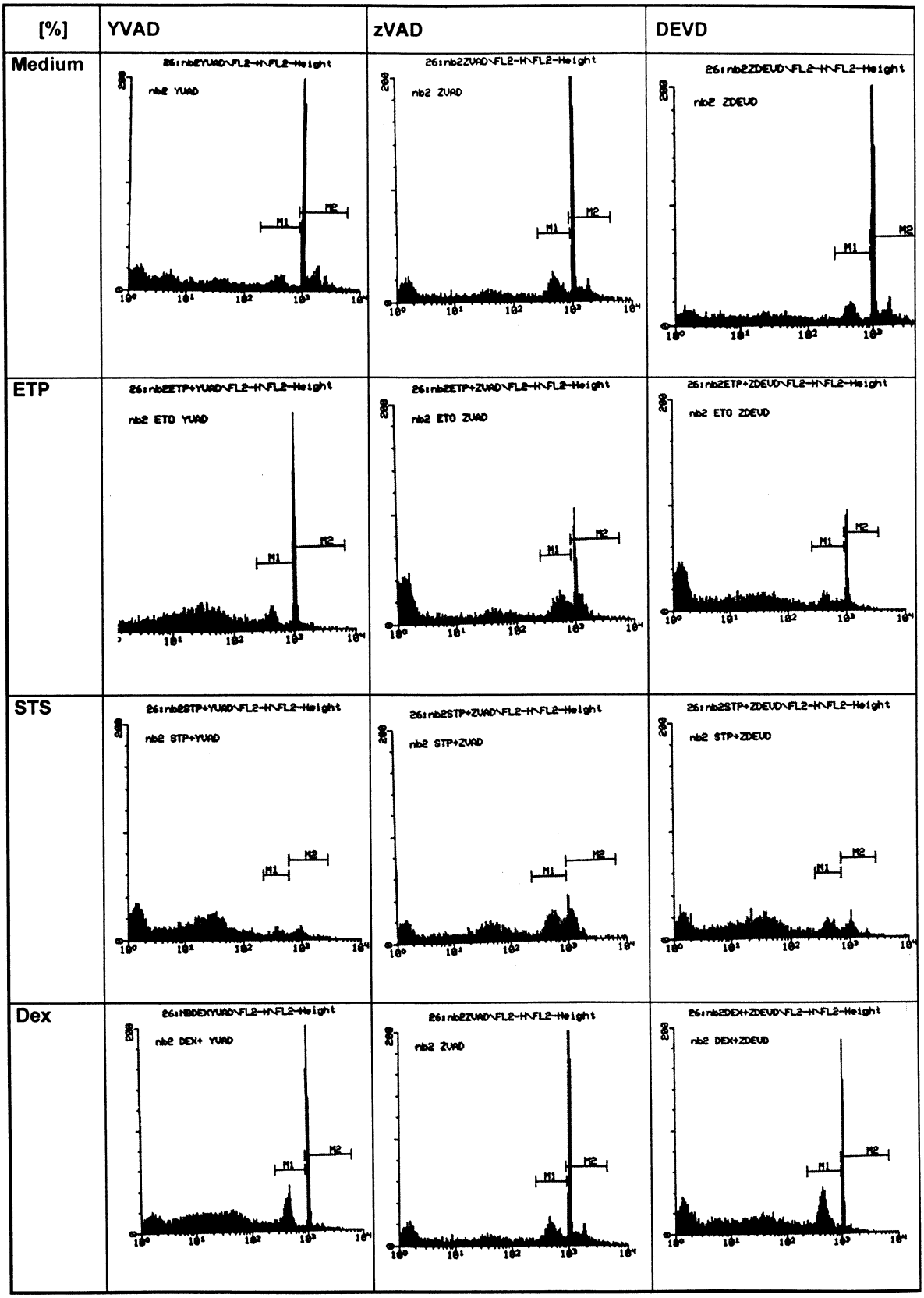

Fig. 3. (*Continued*)

decrease in $\Delta\Psi_m$ (Fig. 3). This indicates that GCs use the activation pathway of a ICE-like protease to induce the fall of the mitochondrial potential. We hypothesise that PRL blocks the $\Delta\Psi_m$ disruption by inhibiting an unknown caspase upstream of the mitochondrial transmembrane potential.

4. Discussion

A striking feature in cells undergoing programmed cell death is the disruption of the mitochondrial transmembrane potential, marked as a point of no return in cell death programming [20]. The mitochondrial membrane has a electrical gradient and is negatively charged on the inner side. The opening of mitochondrial permeability transition (PT) pores is connected with a permeability increase of the inner mitochondrial membrane and mediates the apoptotic process [21]. This causes uncoupling of the respiratory chain, a collapse of $\Delta\Psi_m$, a cessation of ATP synthesis and mitochondrial release of two factors: cytochrome c, a 15 kD protein that acts together with cytosolic factors to induce nuclear apoptosis [22] and AIF, a protease that is characterised as an apoptosis inducing factor [23]. PT is now being postulated as the main candidate [24] for central apoptotic executioner. GCs such as Dex induce the fall of the mitochondrial potential [19]. We could demonstrate that PRL can completely block the mitochondrial permeability transition that is caused by Dex. With the initiation of the decrease of the $\Delta\Psi_m$, the outer membrane exposes PS residues [25]. PRL is capable to inhibit the PS exposure and the total loss of DNA that is induced by GCs. In conclusion, the protective effect of PRL takes place during the induction phase of the apoptotic cascade.

Bcl-2 is recognised to possess protective, anti-apoptotic effects in the process of programmed cell death. Together with other members of the bcl-2 family it determines through homo- or heterodimerisation the fate of the cell [26]. PRL is known to lead to an upregulation of bcl-2 expression in protein and m-RNA

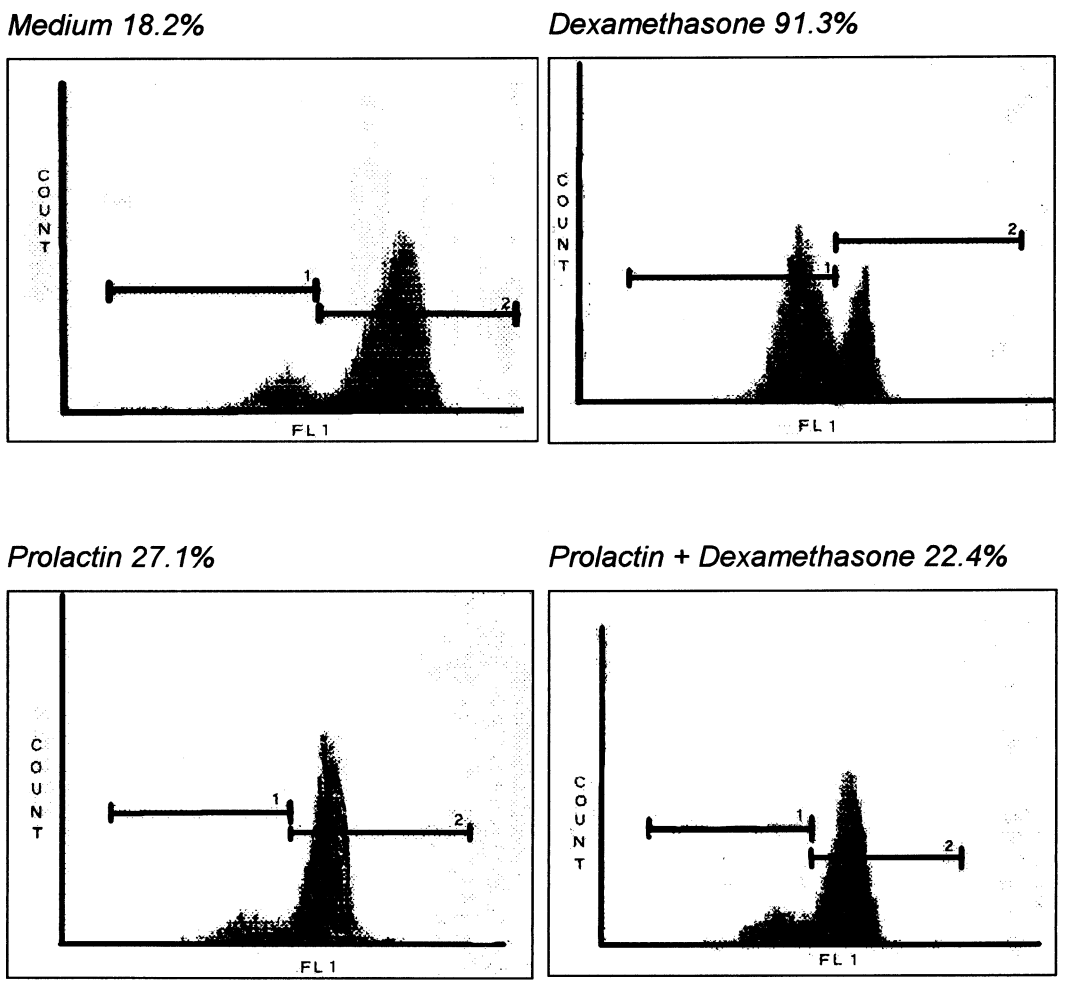

Fig. 4. Measurement of the transmembrane mitochondria potential, $\Delta\Psi_m$. After stimulation with PRL, Dex, PRL + Dex for 18 h Nb_2 cells were collected and stained with $DIOC_6$ and analysed by a flow cytometer. Numbers are referring to the percentage of cells expressing the assessed markers. PRL inhibits Dex induced cell death by blocking the mitochondrial membrane potential $\Delta\Psi_m$.

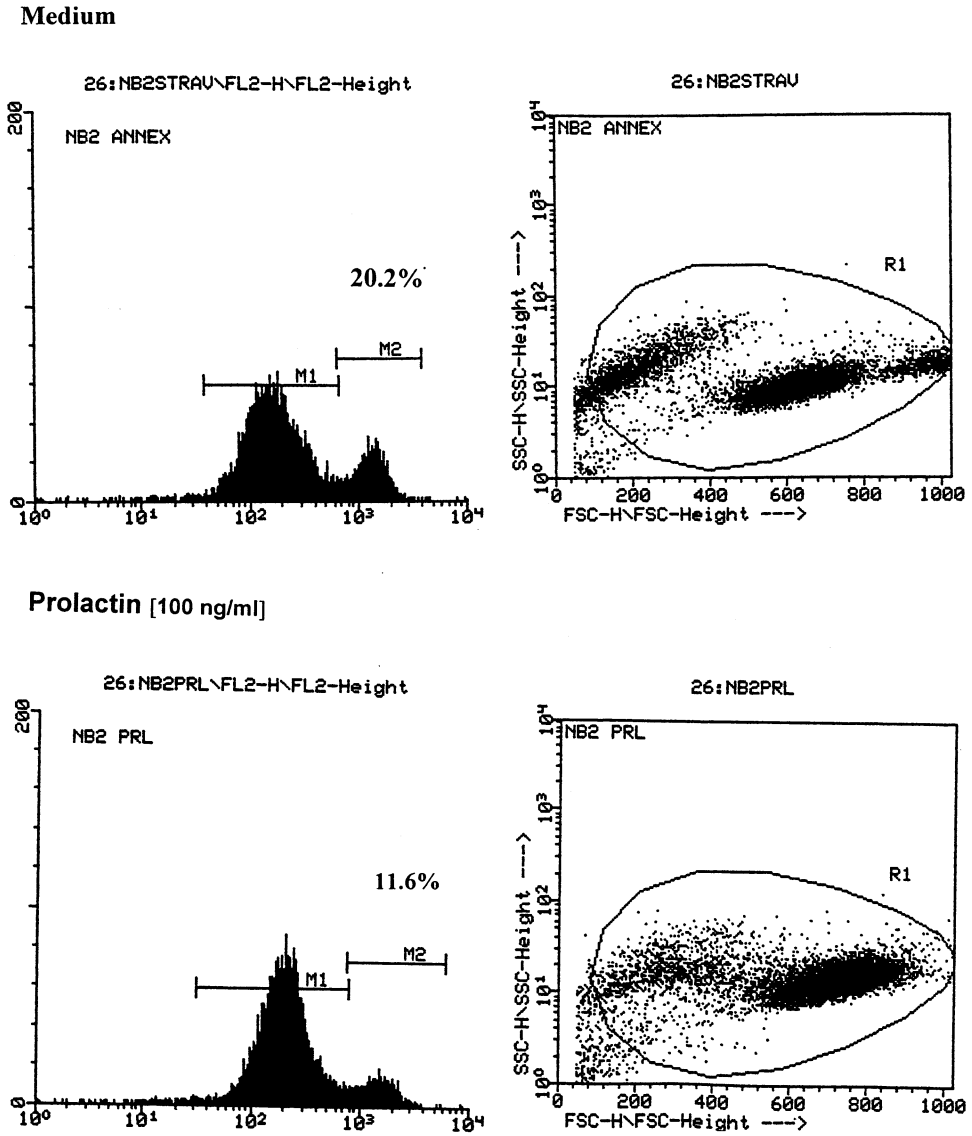

Fig. 5. PS exposure under different conditions. PRL inhibits PS exposure on the outer cell membrane induced by Dex. Numbers are referring to the percentage of apoptotic cells.

levels whereas bax expression is not altered through PRL stimulation. There is a delay between maximal bcl-2 mRNA expression and accumulation of its protein product, indicating the post-transcriptional regulation of bcl-2 [14]. Bag-1, another member of the bcl-2 family decreases its protein levels under Dex treatment. This supposes the idea that bag-1 promotes cellular survival and growth [27]. In our experiments co-stimulation with Dex does not maintain the upregulation of the bcl-2 expression. This finding implicates that bcl-2 is not primarily involved in the protective effect of PRL in Dex induced apoptosis.

zVAD.fmk is an antagonist of ICE-like proteases and acts as an universal inhibitor of nuclear apoptosis in mammalian cells [28]. In addition, AIF has the capacity to trigger the disruption of the mitochondrial membrane and consequently shares a biological effect with ICE [24]. In Nb_2 cells Dex induced disruption of the mitochondrial membrane can be inhibited by the ICE-like inhibitor zVAD.fmk. Caspase 3 family proteases inhibitor zDEVD.fmk and ICE inhibitor YVAD.cmk fail to antagonise the effects. It can be speculated that PRL interacts with an unknown caspase upstream of $\Delta\Psi_m$.

Cysteine proteases are proteolytically activated by the disruption of the mitochondrial membrane and the release of AIF [17]. Caspase 3/Yama/Apopain as a member of this family is synthesised as a proenzyme and is activated proteolytically. CPP32 cleaves subsequently 116 kD PARP into its 85 and 25 kD fragments [29]. PARP may exert a protective function through its involvement in DNA repair and the control of the integrity of the genome [30] but contrary triggers DNA-damage-induced apoptosis. Even if PARP cleavage can

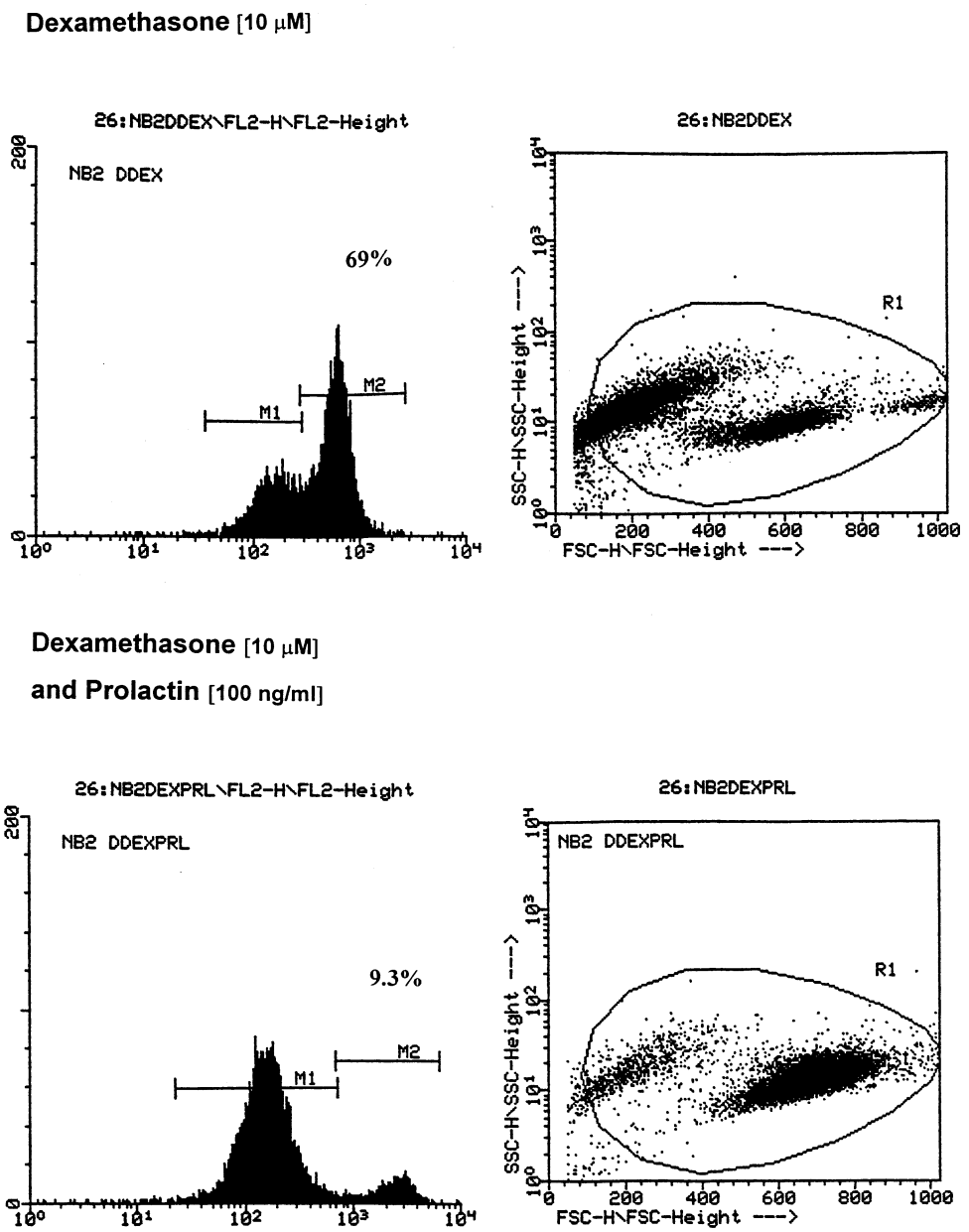

Fig. 5. (*Continued*)

result in DNA fragmentation, the cleavage of ICE-like proteases are an early step in apoptosis and occur before any morphological changes of the cells become visible. Other substrates of the cysteine proteases are lamins, topoisomerases, sterol regulatory element binding protein-1 or the U1-70 kD protein [31]. Estoppey et al. demonstrated that bcl-2 inhibits the activation of CPP32 and proteolysis of PARP and U1-70 kD. Consequently bcl-2 acts upstream of the cysteine proteases [32]. However, CPP32 activation can occur independently of programmed cell death and appears as a physiological step during T lymphocyte activation [33]. We present here that PARP is already cleaved in normally proliferating Nb_2 cells, indicating that PARP

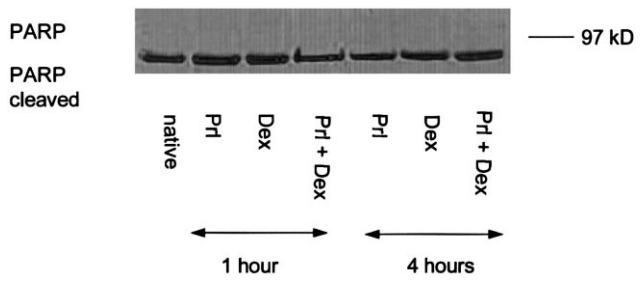

Fig. 6. Western blot of PARP expression in Nb_2 cells. Cells were stimulated for 18 h with Dex (10 μM) and/or oPRL (100 ng/ml).

	cell cycle [%]	$\Delta\Psi_m$ [%]	DEVdase activity [U]
growth medium	1.4	17.0	81.0
starvation medium	4.7	27.0	101.0
PRL [100 ng/m]	1.4	18.7	87.0
Dex [10 μM]	32.7	69.0	100.0
PRL+Dex	1.8	25.0	117.0
DEVD [1 μM]	10.7	25.6	19.5
Dex + DEVD	51.0	64.0	13.0
YVAD [100 μM]	8.0	19.0	105.0
YVAD + Dex	45.3	60.0	111.0

Fig. 7. DEVDase assay, comparison of data obtained by cell cycle analysis, transmembrane mitochondrial potential ($\Delta\Psi_m$) and analysis of DEVDase activity.

cleavage is not exclusively related to Dex induced cell death. This finding is supported by the results of the DEVdase assay.

4.1. Concluding remarks

PRL is a potent inhibitor of apoptosis inducers such as C_2 and Dex. In the present paper we obtain evidence that PRL inhibits the loss of the mitochondrial transmembrane potential that is mediated by GCs: whereas Dex leads to a loss of the transmembrane potential, co-stimulation with PRL can totally block this interruption. These findings indicate that the effect of PRL takes place during the induction phase of the apoptotic cascade. In addition, PRL inhibits the exposure of PS on the outer membrane leaflet as well as the loss of the DNA content induced by Dex. As shown by Castedo et al. [25], cells first undergo the disruption of the mitochondrial membrane potential and then PS exposure occurs. Hence PS exposure only affects cells that already have a low transmembrane potential. We could demonstrate that the known upregulation of bcl-2 is not maintained in the presence of GCs. In conclusion, bcl-2 itself seems not to be the main candidate for the protective effect of PRL. The fall of $\Delta\Psi_m$ leads to an activation of the ICE-like cystein protease CPP32/Yama/Apopain and subsequently cleaves PARP. In normally growing Nb_2 cells PARP is already cleaved and is not exclusively related to Dex induced cell death. Analysis of ICE-like inhibitors such as zVAD.fmk can block Dex induced $\Delta\Psi_m$ disruption in Nb_2 cells. In contrast, the ICE inhibitor YVAD as a caspase-3 family inhibitor zDEVD are not capable to prevent the GC induced fall of the mitochondrial transmembrane potential. Further studies will elucidate if PRL blocks GC induced $\Delta\Psi_m$ disruption by inhibiting an unknown caspase.

Acknowledgements

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