6. Methods

6.1 Cell culture

6.1.1 Growth media and solutions

For growth and cultivation of melanoma cells following media and solutions were used.

Dulbecco´s modified Eagle medium (DMEM / 10 % FCS)

DMEM (incl. 4 mM L-glutamine, 25 mM glucose; without sodium pyruvate)	500	ml		
heat-inactivated foetal calf serum (FCS)	50	ml		
10,000 IE penicillin /	5	ml	168	µM
10,000 µg/ml streptomycin stock solution			69	µM

The complemented medium can be stored for four weeks at 4 °C.

Roswell Park Memorial Institute 1640 medium (RPMI 1640 / 10 % FCS)

RPMI 1640	500	ml		
heat-inactivated foetal calf serum (FCS)	50	ml		
10,000 IE penicillin /	5	ml	168	µM
10,000 µg/ml streptomycin stock solution			69	µM
200 mM L-glutamine	5	ml	2	mM

The complemented medium can be stored for four weeks at 4 °C.

Trypsin solution

trypsin	1.5	g	126	µM
phosphate buffered saline (PBS)	500	ml		

The solution is stored as aliquots of 10 ml at -20 °C.

Freezing solution for melanoma cells

DMEM	2	ml
heat-inactivated foetal calf serum (FCS)	2	ml
dimethylsulfoxid	1	ml

Freezing solution for HaCaT cells

heat-inactivated foetal calf serum (FCS)	4.5	ml
dimethylsulfoxid	0.5	ml

6.1.2 Cultivation of cells

Melanoma cells are grown in 12 ml DMEM / 10 % FCS in 75 cm^2 tissue culture flasks in a vaporised atmosphere with 5 % carbon dioxide at 37 °C. All melanoma cell lines grow adherently on the ground of the flasks. Every third day the medium is aspirated, cells are rinsed with 6 ml PBS and supplemented with fresh medium to remove dead cells and catabolites.

For passaging, confluent cells are washed two times with 6 ml PBS and then incubated with 3 ml trypsin solution for 10 min at 37 °C. Due to the loss of cell-cell contacts the cells begin to round and detach from the ground. The cells are transferred into 9 ml DMEM / 10 % FCS. The high protein content of the FCS stops the proteolysis by the trypsin. After centrifugation at 150 ×g for 5 min, the supernatant is aspirated and the cells resuspended in DMEM / 10 % FCS. The cells are counted in a hemocytometer and for maintenance purpose the cells are seeded with 10,000 cells/cm^2 in new flasks. The first change of medium occurs after 36 h.

HaCaT cells are cultivated essentially as described for melanoma cells but instead of DMEM / 10 % FCS, RPMI 1640 / 10 % FCS is used.

HL60 cells are growing in suspension in RPMI 1640 / 10 % FCS. Cells are supplied with one third of the actual volume with fresh medium every third day. When a density of > 1,000,000 cells/ml is reached, the cells are diluted to 500,000 cells/ml into new flasks.

6.1.3 Freezing and thawing of cells

Confluent cells are washed two times with 6 ml PBS and then incubated with 3 ml trypsin solution for five to ten minutes at 37 °C. The cells are transferred into 9 ml of the respective medium and pelleted at 150 ×g for 5 min. The supernatant is aspirated and the cells resuspended in 1 ml of respective freezing solution. The addition of DMSO avoids crystal growth within the cells. Freezing of cells should be a slow process, therefore, the tubes are packed into tissue paper and frozen at -20 °C for six hours. Subsequently, the cells are relocated to -80 °C and can be stored for up to six months or are transferred after two days into liquid nitrogen. At -196 °C the cells can be stored unlimited.

On the contrary, thawing of cells should be fast. Therefore, the cells are thawed at 37 °C in a water bath, transferred into 20 ml of the respective medium prewarmed to 37 °C and centrifuged at 150 ×g for 5 min. The pellet is resuspended in 24 ml of prewarmed medium and dispensed into two flasks.

6.2 Lipid chemistry

6.2.1 Preparation of substrate vesicles for the *in vitro* phospholipase D assay

Solutions used

Vesicle buffer

HEPES	1.192	g	50	mM
EGTA	0.114	g	3	mM
potassium chloride	0.596	g	80	mM
sodium hydroxide	ad pH 7.5			
water, double-distilled	ad 100	ml		
250 mM 1,4-dithiothreitol (add directly before use)	400	µl	1	mM

For ten reaction mixtures, following lipids in chloroform are mixed and dried with a nitrogen flow.

			concentration in the assay	
1 µg/µl phosphatidylethanolamine from bovine brain (60 % plasmalogen)	27.9	µl	ca. 140	µM
1 mM phosphatidylinositol- 4,5-bisphosphate	3.60	µl	12	µM
463 µM 1-palmitoyl-2-[1-^{14}C]-palmitoyl-L-3-phosphatidylcholine	5.57	µl	8,6	µM

To this mixture, 50 µl of vesicle buffer are added and, for rehydration of the lipids incubated on ice for 1 h. Vesicle formation is achieved by sonication in an ultrasonic bath for 10 min. Substrate vesicles, 5 µl each are added immediately to the reaction mixtures (see chapter 6.3.1).

6.2.2 Identification and quantification of radioactively labelled lipids

Solutions used

Separation solvent system (BROWN et al., 1993)

chloroform	39	ml	9.5	M
methanol	9	ml	4.4	M
glacial acetic acid	3	ml	1.0	M
	13:3:1	v/v/v		

Samples dried with nitrogen are resuspended in chloroform and applied to silica gel 60 HPTLC plates using an automatic sample applicator. The plates are developed vertically with the solvent system according to BROWN *et al*. (1993). Dried plates are wrapped in cellophane and exposed to phosphorimager plates for 7 h. For calibration, enzyme assays as described in chapter 6.3.1 were performed using 5 units peanut phospholipase D without and with ethanol or with 5 units phospholipase C. The derived lipid mixtures contain besides the substrate

1-palmitoyl-2-[1-^{14}C]-palmitoyl-L-3-phosphatidylcholine radioactively labelled phosphatidic acid, phosphatidic acid and phosphatidylethanol or diacylglycerol, respectively.

6.3 Enzyme reactions

6.3.1 Assay of phospholipase D1 activity

Phospholipase D1 is assayed essentially as described by BROWN *et al.* (1995). Cell lysates are incubated with substrate vesicles containing phosphatidylethanolamine, phosphatidylinositol-4,5-bisphosphate and phosphatidylcholine in a molar ratio of 16:1.4:1 and ethanol as substrates. Phospholipase D generates via its transphosphatidylation activity phosphatidylethanol from phosphatidylcholine and ethanol (YANG *et al.*, 1967). The lipids are separated by thin layer chromatography and the bands are quantified (see chapter 6.2.2).

Solutions used

Assay buffer (2×)

HEPES	11.92	g	100	mM
EGTA	1.141	g	6	mM
potassium chloride	5.965	g	160	mM
magnesium chloride hexahydrate	0.610	g	6	mM
calcium chloride dihydrate	0.588	g	4	mM
sodium hydroxide	ad pH 7.5			
water, double-distilled	ad 500	ml		

A standard assay contains			final concentration	
250 mM 1,4-dithiothreitol	0.1	µl	1	mM
protein	24	µg	0.8	µg/µl
ethanol	0.7	µl	0.4	M
assay buffer	ad 25	µl		

Stimulators, such as guanosine 5'-*O*-(3-thiotriphosphate) and 12-*O*-tetradecanoyl phorbol-13-acetat or inhibitors such as sodium oleate are included in the reaction mixture as indicated. The reaction is started by addition of 5 µl of substrate vesicles (see chapter 6.2.1) and incubated for 60 min at 37 °C. Subsequently, 120 µl of chloroform/methanol 2:1 (v/v) and 30 µl of double-distilled water are added and mixed for 5 sec. Phase separation is achieved by centrifugation for 5 min at 13,000 ×g. The chloroform-phase is transferred into a new tube and the solvent is evaporated in an oxygen flow for 5 min. Separation and quantification of lipids are performed as described in chapter 6.2.2.

6.4. Protein chemistry

6.4.1 Preparation of cell lysates and subcellular fractionation

Solutions used

Lysis buffer

assay buffer (2×)	500	µl		
250 mM 1,4-dithiothreitol	4	µl	1	mM
1 mM leupeptin	10	µl	10	µM
10 mM pepstatin	1	µl	10	µM
0.1 M phenylmethanesulfonyl fluoride	10	µl	1	mM
water, double-distilled	ad 485	µl		

Cells are washed twice with PBS and scraped off in about 8 µl/cm^2 of lysis buffer on ice. The lysate is homogenised by 15 strokes in a glass-glass dounce homogeniser on ice and centrifuged twice for 5 min at 4 °C with 400 ×g. The resulting post-nuclear supernatant is used directly as whole cell lysate or further processed for subcellular fractionation. Therefore, the whole cell lysate is centrifuged for 1 h at 4 °C with 100,000 ×g. The supernatant is used as cytosolic fraction and the pellet is resuspended in lysis buffer and used as membrane fraction.

6.4.2 Determination of protein concentration

The determination of protein concentrations using bicinchoninic acid is based on a method described by SMITH *et al.* (1985). Copper(II) forms in the biuret reaction in alkaline milieu a red- to blue violet complex with the amide nitrogen atoms of the peptide bonds. Within this complex the copper(II) is reduced to copper(I). One copper(I)-cation forms a stable chelate complex of violet colour with two molecules of bicinchoninic acid. This complex exhibits an absorption maximum at 562 nm that is used for quantification with a photometer. Depending on the incubation temperature different sensitivities can be achieved.

incubation time	temperature	concentration range
30 min	60 °C	5 - 250 µg/ml
30 min	37 °C	20 - 1200 µg/ml
2 h	room temperature	20 - 1200 µg/ml

This assay is superior to the method by LOWRY *et al.* (1951) because of easy handling, the stability of the working reagent up to one week and the low influence of detergents and salts.

Solutions used

Solution A

disodium bicinchoninate	10	g	26	mM
disodium carbonate monohydrate	20	g	161	mM
disodium tatrate dihydrate	1.6	g	7	mM
sodium hydroxide	4.0	g	100	mM
sodium hydrogencarbonate	9.5	g	113	mM
water, double-distilled	ad 1,000	ml		

Solution A is purchased from Pierce (Weiskirchen, D).

Solution B

copper(II) sulphate pentahydrate	1	g	160	mM
water, double-distilled	ad 25	ml		

The solution is sterile filtrated.

Both solutions are stable infinitely at room temperature.

The working reagent is prepared by adding one part of solution B to fifty parts of solution A. In a 96-well microtitre plate 2 µl of the sample and 8 µl of double-distilled water are mixed. A standard rank is prepared in parallel by each 2 µl of sample buffer and 8 µl of suitable concentrations of bovine serum albumin in double-distilled water. The dilution of the samples and the inclusion of sample buffer with the standard rank is required. The sample buffer contains leupeptin and pepstatin which are peptides and therefore undergo the biuret reaction, whereas phenylmethansulfonyl fluoride and dithiothreitol interfere as reducing agents. Each sample and standard is pipetted at least in triplets. To the wells, 200 µl of working reagent are added, mixed and incubated as described. The absorption is measured at 570 nm in an ELISA photometer. With the standard rank a regression of the form $A = a + b \cdot e^{-cP}$, where A is the absorption, P is the protein concentration and a, b and c are the variables, is performed. The protein concentration of the sample is calculated using the obtained values of the variables.

6.4.3 Discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis

The discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is performed in slightly modified form as described by LAEMMLI (1970). Polyacrylamide gels are prepared by free radical-induced polymerisation of acrylamide with *N*,*N*'-methylenebisacrylamide as cross linker. Ammonium persulphate (APS) and *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TEMED) are added as provider and stabiliser of free radicals, respectively. All buffers contain the anionic detergent sodium dodecyl sulphate (SDS) which binds quite tenaciously to proteins causing them to denature. Most proteins bind

SDS in the same ratio of 1.4 g of SDS per 1 g of protein, that is about one SDS molecule for every two amino acid residues. Dithiothreitol is added to the sample buffer to reduce disulphide bonds. In contrast to mercaptoethanol, the latter does not tend to form bonds with sulfhydryl groups. The large negative charge that the SDS imparts masks the protein's intrinsic charge so that complexes of identical charge-to-mass ratios are formed. Consequently, the denatured proteins are separated on electrophoresis in order of their molecular masses because of gel filtration effects. The size of unknown proteins is determined by running markers of known size in parallel.

Solutions used

AA-BIS

acrylamide	386.6	g	5.44	M
N,*N*'-methylenebisacrylamide	13.4	g	87	mM
water, double-distilled	ad 1,000	ml		

The ratio acrylamide:*N*,*N*'-methylenebisacrylamide is 29:1 (w/w) or 40 %T and 3,4 %C.

10 % APS

ammonium persulphate	0.1	g	0.44	M
water, double-distilled	ad 1	ml		

The solution is stable for four weeks at 4 °C.

Running gel buffer (5×)			final concentration	
Tris	113.5	g	375	mM
sodium dodecyl sulphate	2.5	g	35	mM
hydrochloric acid	ad pH 8.8			
water, double-distilled	ad 500	ml		

Stacking gel buffer (4×)			final concentration	
Tris	16.95	g	175	mM
sodium dodecyl sulphate	0.8	g	35	mM
hydrochloric acid	ad pH 6.8			
water, double-distilled	ad 200	ml		

Electrophoresis buffer (10×)			final concentration	
Tris	60.55	g	175	mM
sodium dodecyl sulphate	20.19	g	35	mM
glycine	285.27	g	190	mM
water, double-distilled	ad 200	ml		

The pH-value should not be adjusted and should be pH 8.3.

Loading buffer (5×)			final concentration	
1 M Tris/HCl pH 6.8	750	µl	30	mM
glycerol	1,250	µl	0.7	M
sodium dodecyl sulphate	0.25	g	35	mM
1,4-dithiothreitol	0.39	g	0.1	M
1 % bromophenol blue	250	µl	0.14	mM
water, double-distilled	ad 5	ml		

Pipetting scheme for two gels of the size 80 mm × 60 mm × 1.5 mm.

	running gel 6 %T	stacking gel 2 %T	running gel 15 %T	stacking gel 3 %T
water, double-distilled [ml]	11.6	5.54	8.4	5.34
running gel buffer (5×) [ml]	3.6	-	4	-
stacking gel buffer (4×) [ml]	-	2	-	2
AA-Bis [ml]	2.7	0.4	7.5	0.6
TEMED [µl]	5	6	5	6
10 % APS [µl]	95	55	95	55

The running gel solution is prepared as given in the table above. Polymerisation starts with the addition of TEMED and ammonium persulphate. The carefully mixed solution is poured into the sandwich of the slab gel unit, leaving enough space for the stacking gel with comb, and overlaid with water-saturated 1-butanol. When polymerisation has completed after about 30 min, the overlay is poured off, washed with running gel buffer and completely removed with the edge of a Whatman 3MM paper. The sandwich is filled with stacking gel solution and a comb is inserted. After completion of polymerisation, the slab gel unit is integrated into the electrophoresis chamber, filled with electrophoresis buffer and the combs are removed. Each well is then rinsed with electrophoresis buffer.

The samples are adjusted to equal protein concentrations using the corresponding lysis buffer and one fourth of the volume sample buffer (5×) is added. Routinely, samples are denatured for 5 min at 95 °C but samples intended for detection of phospholipase D1 are denatured for 30 min at 37 °C. This procedure is required due to the posttranslational fatty acylation of phospholipase D1 that results in aggregation and precipitation at higher temperatures (SUGARS *et al.*, 1999).

Electrophoresis is performed from cathode to anode at 35 mA per gel for 15 min and subsequently at 20 mA per gel with voltage limited to 150 V. When the bromophenol blue reaches the bottom of the gel, the electrophoresis is terminated. The sandwiches are disassembled and the gels subjected to further processing.

6.5 Immunochemistry

6.5.1 Purification of antibodies

Peptide-specific antibodies can be purified by affinity chromatography using the peptides as antigen. The resulting immune complex can be dissociated only by strong means like chaotropic salts or low pH which often cause loss of binding activity. For maximal recovery and preservation of antibodies a method using high salt concentrations and glycol at neutral pH as eluant was applied (TSANG and WILKINS, 1991).

6.5.1.1 Preparation of affinity columns

The affinity columns were prepared using the SulfoLink® Kit from Pierce (Weiskirchen, D). Immobilised iodoacetyl groups on a crosslinked agarose support are used which specifically react with sulfhydryl groups.

Solutions used

Coupling buffer

Tris	3.03	g	50	mM
sodium EDTA	0.93	g	5	mM
hydrochloric acid	ad pH 8.5			
water, double-distilled	ad 500	ml		

The storage solution is drained from the column. Then, the column is equilibrated with 12 ml of coupling buffer and subsequently 3 ml of a 2 mM solution of the cysteine-containing peptide in coupling buffer are added. The column is mixed for 15 min at room temperature. After a further incubation for 30 min at room temperature without mixing, the column is washed with 6 ml of coupling buffer. Non-specific binding sites are blocked by incubation with 2 ml of 50 mM cysteine for 15 min at room temperature under mixing. After a further incubation for 30 min at room temperature without mixing, the column is washed four times with 4 ml of 1.0 M sodium chloride and four times with 10 mM sodium azide. After insertion of the top porous disc, 2 ml of degassed 10 mM sodium azide are added and the column is stored at 4 °C. Coupling efficiency is calculated after measurement of the absorption of eluates at 280 nm.

6.5.1.2 Affinity chromatography

Solutions used

Sample buffer

TBS (10×)	4	ml		
0.1 M PMSF	400	µl	1	mM
10 mM leupeptin	40	µl	10	µM
5 M sodium azide	80	µl	10	mM
water, double-distilled	ad 40	ml		

Incubation buffer

sample buffer	2	ml
bovine serum albumin	2	mg

Elution buffer

1 M HEPES, pH 8.0 NaOH	0.45	ml	75	mM
magnesium chloride hexahydrate	3.66	g	3	M
1,2-ethanediol	1.5	ml	4.5	M
water, double-distilled	ad 6	ml		

Washing buffer

HEPES	5.96	ml	50	mM
sodium thiocyanate	243.21	ml	6	M
sodium hydroxide	ad pH 7.2			
water, double-distilled	ad 500	ml		

After draining the storage solution, the column is washed with 6 ml of sample buffer. Meanwhile, 500 µl of αNChPLD1 antiserum are added to 500 µl of incubation buffer and incubated for 5 min at room temperature. The sample is added to the column and after entering the gel 200 µl of incubation buffer are applied. For blocking of the column before the first run, a solution of 10 mg bovine serum albumin and 10 mg lysozyme in sample buffer is used instead of sample. The flow through is stopped, 1 ml of incubation buffer is added and the column is incubated for 1 h at room temperature. Subsequently, the column is washed five times with 6 ml of sample buffer. Antibodies are eluted using three times 2 ml of elution buffer. The eluate is collected on Centriplus YM-30 filter columns on ice. Regeneration of the affinity column is achieved by washing with 40 ml of wash buffer, 40 ml of PBS and 16 ml of 10 mM sodium azide. The eluate is centrifuged for 16 h at 4 °C with 3000 ×g. Subsequently, the filter is washed twice by application of 12 ml of PBS-T and centrifugation for 2 h at 4 °C with 3000 ×g. A further wash with twice 12 ml of PBS containing 10 mM sodium azide succeeds. For elution of the antibodies, the filter column is inverted and centrifuged for 4 min

at 4 °C with 2000 ×g. The final protein concentration is adjusted to ≥ 0.25 mg/ml by adding bovine serum albumin.

6.5.2 Denaturing immunoprecipitation

Proteins can be precipitated using a specific antibody if protein A coupled to a solid matrix is used to immobilise the immunocomplex. The denaturing immunoprecipitation includes SDS with the formation of the immunocomplex. Antibodies made against oligopeptides most likely recognise the denatured epitope.

Solutions used

DIP buffer

assay buffer (2×)	10	ml		
100 mM sodium vanadate	200	µl	1	mM
100 mM sodium fluoride	10	µl	50	µM
0.1 M PMSF	200	µl	1	mM
10 mM leupeptin	200	µl	0.1	mM
10 mM pepstatin	20	µl	10	µM
10 % sodium dodecyl sulphate	200	µl	3.5	mM
5 % sodium deoxycholate	2	ml	12	mM
Triton X-100	200	µl	1	%
water, double-distilled	ad 20	ml		

The cells are washed twice with PBS, scraped off with DIP buffer and homogenised by 15 strokes in a dounce homogeniser. After centrifugation for 5 min at 4 °C with 13000 ×g, 800 µg of the supernatant are diluted to a final volume of 800 µl with DIP buffer. Protein A sepharose, 10 mg are swollen in 1 ml of PBS for 10 min at 4 °C. The protein A sepharose is washed by centrifugation for 20 sec at 4 °C with 13000 ×g, resuspending the pellet in 500 µl of DIP buffer, centrifugation and resuspending the pellet in 200 µl of DIP buffer. Of the protein A sepharose suspension, 100 µl are added to the homogenate and incubated for 1 h at 4 °C under rotation. After centrifugation for 20 sec at 4 °C with 13000 ×g the supernatant is supplied with 4 µl of αNChPLD1 and incubated for 1 h at 4 °C under rotation. Subsequently, 100 µl of protein A-sepharose suspension are added and the solution is incubated for 16 h at 4 °C under rotation. After centrifugation for 20 sec at 4 °C with 13000 ×g, the precipitate is washed three times by resuspending with 1 ml of DIP buffer, incubation for 20 min at 4 °C under rotation and subsequent centrifugation. The precipitate is then resuspended with 30 µl of SDS-PAGE loading buffer (5×) and incubated for 5 min at 95 °C. To clear the solution

from sepharose, a hole is pierced into the bottom of the tube using a hot needle and the solution is centrifuged for 2 min with 13000 ×g into a new tube.

6.5.3 Western blotting

Distinct proteins separated by SDS-PAGE can be detected via binding of specific antibodies. An antigen-antibody reaction inside the gel is always accompanied by loss of resolution when possible at all. For this reason, the proteins are transferred onto a membrane using an electrical field (TOWBIN *et al.*, 1979). In general, the proteins are negatively charged at the transfer pH due to the bound SDS and will transfer from the cathode to the anode. The immobilised proteins can then be probed with antibodies on the membrane.

Solutions used

Transfer buffer (2×)

			final concentration	
Tris	12.11	g	25	mM
glycine	28.83	g	192	mM
methanol	800	ml	4.9	M
water, double-distilled	ad 2,000	ml		

The pH-value should not be adjusted and should be pH 8.3.

Staining solution

ponceau S	10	g	13	mM
glacial acetic acid	50	ml	0.9	M
water, double-distilled	ad 1,000	ml		

Fixing solution

glacial acetic acid	5	ml	0.9	M
water, double-distilled	ad 100	ml		

Western blotting with a tank transfer system follows immediately the SDS-PAGE. Nitrocellulose membrane is wetted in double-distilled water and subsequently, together with sponges and 3MM paper, soaked in transfer buffer for 5 min. The transfer sandwich is assembled under transfer buffer. On the black side of the cassette a sponge, a 3MM paper, the gel, the membrane and another 3MM paper are laid. Now, air bubbles are removed by rolling a test tube over the sandwich. A second sponge is laid on top and the cassette is assembled and inserted into the transfer tank with the membrane facing the anode. The tank is filled with transfer buffer to completely cover the electrode panels. A magnetic stirrer is used to stir the buffer and the tank is put into ice for cooling. Gels of 6 %T are blotted for 1.5 h and gels of 15 %T are blotted for 45 min at 100 V with current limited to 250 mA. Efficient binding of

protein is confirmed by reversible staining using ponceau S. The membrane is incubated in staining solution for 2 min and molecular-weight markers are marked with a pencil.

6.5.4 Immunodetection of blotted proteins

Following the Western blotting, the immobilised proteins are probed with primary antibodies specific for the sample protein followed by secondary antibodies specific for the general class of primary antibodies. A peroxidase reporter enzyme is coupled to the secondary antibodies and the antigens are visualised with chromogenic substrates for peroxidase. Here, a chemiluminescence reaction is used. Peroxidase can reduce hydrogen peroxide to water using luminol as reducting agent. An enhancer enhances and stabilises the elevated state of the luminol aquired by the reduction. Upon return to the ground state, light is emitted that is detected using an X-ray film.

Solutions used

PBS (10×)

			final concentration	
potassium chloride	4	g	2.68	mM
potassium dihydrogenphosphate	4	g	1.47	mM
sodium chloride	160	g	137	mM
disodium hydrogenphosphate dihydrate	28.8	g	8.09	mM
water, double-distilled	ad 2,000	ml		

The pH-value should be pH 7.4 and is not adjusted. The osmolarity is about 300 mosmol. This PBS is not used for cell culture.

The following volumes are adjusted for treatment of one membrane.

PBS-T

PBS (10×)	50	ml		
Tween 20	500	µl	0.1	%
water, double-distilled	ad 500	ml		

Blocking buffer A

skim milk powder	0.1	g	1	%
PBS-T	10	ml		

Blocking buffer B

skim milk powder	0.3	g	3	%
PBS-T	10	ml		

Blocking buffer C

skim milk powder	0.1	g	1	%
bovine serum albumin	0.1	g	1	%
PBS-T	10	ml		

Super Signal working solution

luminol / enhancer solution	3	ml
stable peroxide solution	3	ml

The working solution is stable for approximately 24 h. The luminol / enhancer solution and the stable peroxide solution are purchased from Pierce (Weiskirchen, D). No information about the ingredients is given by the supplier.

Stripping buffer

glycine	15	g	0,2	M
sodium dodecyl sulphate	10	g	35	mM
Tween 20	1	ml	0,1	%
hydrochloric acid	ad pH 2.3			
water, double-distilled	ad 1000	ml		
5 M sodium azide (add directly before use)	2	ml	10	mM

The membrane is washed two times using double-distilled water and then incubated in 10 ml of blocking buffer for 20 min at room temperature under gentle agitation. The blocking buffer is discarded, and primary antibody diluted in 10 ml of blocking buffer as indicated in the table below is added to the membrane with consequent incubation for 2 h at room temperature under gentle agitation. Alternatively, incubation can be performed at 4 °C over night.

primary antibody	blocking buffer	primary antibody dilution	secondary antibody dilution
anti actin (mouse)	A	1:500	1:40,000
anti ADP-ribosylation factor (rabbit)	A	1:500	1:10,000
anti caspase-3 (rabbit)	B	1:2,000	1:5,000
anti phospholipase D1 (rabbit)	C	1:1,000	1:10,000
anti protein kinase Cα (mouse)	A	1:500	1:40,000
anti rhoA (mouse)	A	1:1,000	1:40,000

Afterwards, the membrane is washed twice with 15 ml PBS-T for 3 min. To the membrane the secondary antibody diluted in 10 ml of blocking buffer as indicated in the table is added and incubated for 1 h at room temperature under gentle agitation. Washing the membrane twice with 15 ml PBS-T for 3 min, once with 25 ml PBS-T for 6 min, and twice with 15 ml PBS for 2 min follows. For the chemiluminescence reaction the membrane is incubated in Super Signal working solution for 4 min at room temperature under gentle agitation. Subsequently, the membrane is transferred into a plastic membrane protector and air bubbles are removed. An X-ray film is exposed to the membrane for varied periods. To reprobe the

membrane with different antibodies, the blot is washed with PBS and incubated in stripping buffer for 16 h at room temperature.

6.5.5 Immunohistochemical detection using alkaline phosphatase anti alkaline phosphatase antibody (APAAP) complexes

To detect the antigen histochemically one standard method is the labelling with alkaline phosphatase anti alkaline phosphatase antibody (APAAP) complexes (CORDELL *et al.*, 1984). After fixation and incubation with the primary antibodies, unlabelled bridge antibodies are used to detect the primary antibodies. These bridge antibodies also detect the preformed APAAP complexes. This method was developed to detect murine immunoglobulins and commercially available APAAP complexes are made of murine anti alkaline phosphatase antibodies. To detect primary rabbit antibodies further bridging mouse anti-rabbit antibodies are required. The staining is achieved using new fuchsin in combination with levamisole to suppress endogenous phosphatases.

Solutions used

TBS

Tris	4.5	g	7.4	mM
Tris·HCl	34.25	g	43.5	mM
sodium chloride	43.90	g	150	mM
water, double-distilled	ad 5000	ml		

The pH-value should not be adjusted and should be pH 7.4 to pH 7.6.

RPMI dilution

RPMI	50	ml		
foetal calf serum	50	ml	10	%
sodium azide	0.5	g	15	mM
water, double-distilled	450	ml		
hydrochloric acid	ad pH 7.4 to pH 7.6			

Conjugation buffer

RPMI dilution	2	ml		
human serum	250	µl	12.5	%

137 mM New fuchsin

new fuchsin	1	g	137	mM
2 M hydrochloric acid	20	ml		

Nitrite/fuchsin

sodium nitrite	10	mg	0.58	M
water, double-distilled	250	µl		
137 mM new fuchsin	100	µl	39	mM

After addition of the new fuchsin, the solution is mixed for 1 min and immediately added to the fuchsin solution (see below).

NAB phosphate

naphthol AS-BI phosphate	250	mg	184	mM
N,*N*-dimethylformamide	3	ml		

Fuchsin solution

TBS	35	ml		
1,2-propanediol	12.5	ml	4.88	M
hydrochloric acid	ad pH 9.75			
levamisole	20	mg	2.4	mM
nitrite/fuchsin	350	µl		
NAB phosphate	300	µl	1.6	mM
hydrochloric acid	ad pH 8.8			

The solution is prepared freshly and sterile filtrated before use.

The cells are grown on chamber slides and for fixation the medium is removed and the slides incubated in acetone for 10 min at room temperature. After evaporation of the acetone at room temperature the slides can be stored dry at -70 °C for up to one month. All following steps are performed in a humidified chamber at room temperature. The αNChPLD1 antiserum is diluted 1:30 in 1 % bovine serum albumin in PBS and incubated for 5 min. This solution is added to the dried slide and incubated for 30 min. After three washes with TBS for 5 min mouse anti-rabbit antibodies diluted 1:50 in conjugation buffer are added and incubated for 30 min. Following a further three washes with TBS for 5 min the slides are incubated with rabbit anti-mouse antibodies diluted 1:50 in conjugation buffer for 30 min. The slides are washed three times with TBS for 5 min and then incubated with APAAP complexes diluted 1:50 in RPMI dilution for 30 min. Staining is achieved after three washes with TBS for 5 min by incubation in fuchsin solution and subsequent counterstaining with hematoxylin solution for 1 min. After washing with water for 5 min, the preparation is covered with aquamount (Promochem, Wesel, D) and cover glass.

6.6 Molecular biology

6.6.1 Isolation of total RNA

The purification of RNA is performed using kits from Qiagen (Hilden, D). The method is based on the fact that nucleic acids bind to silica beads under distinct ion conditions in alcoholic solutions (VOGELSTEIN and GILLESPIE, 1979). Under the same conditions proteins and carbohydrates can be washed away. The used buffers and disposable materials constitute contents of the kit and no further information is given by the supplier.

Ribonucleases (RNases) are very stable and active enzymes. Inactivation of RNases requires strong modifying agents such as diethylpyrocarbonate (DEPC) or β-mercaptoethanol. Materials, if not purchased as RNase-free, are heat treated for 4 h at 220 °C if suitable, or alternatively incubated overnight in 1 % DEPC and subsequently autoclaved at 121 °C.

Solutions used

RLT, RW1, and RPE buffer are ready-to-use solutions

RLT buffer*

RLT buffer	2.2	ml		
2-mercaptoethanol	22	µl	142	mM

RPE buffer*

RPE buffer	1,475	µl		
ethanol	5	ml	13.7	M

RNase-free water

water, double distilled	1,000	ml		
diethylpyrocarbonate	1	ml	7	mM

The solution is incubated for 12 h at room temperature and subsequently autoclaved at 121 °C.

Cells grown in 9.6 cm^2 wells are lysed in 350 µl RLT* buffer and scraped using an inverted 1 ml pipette tip. The lysate is stored at -20 °C or transferred onto a QIAshredder column and centrifuged for 2 min at 13,000 ×g. The homogenate is mixed with 350 µl of 70 % ethanol and loaded onto a RNeasy spin column. After centrifugation for 30 sec at 13,000 ×g, the flow-through is discarded. The column is washed with 700 µl of RW1 buffer, set into a new test tube and washed with 500 µl of RPE* buffer. Another 500 µl of RPE* buffer are added onto the column and centrifuged for 1 min at 13,000 ×g. The flow-through is discarded and another centrifugation for 1 min at 13,000 ×g follows to remove any remaining ethanol. The RNA is

eluted with 30 µl of RNase-free water by centrifugation for 1 min at 13,000 ×g. The eluate is stored at -20 °C.

6.6.2 Determination of nucleic acid concentration

The concentration of aqueous nucleic acid solutions can be determined by the absorption of UV-radiation of 260 nm wavelength. An absorption (A_{260}) of 1.00 equals a concentration of 50 µg/ml of double-stranded DNA, 33 µg/ml of single-stranded DNA, or 40 µg/ml of RNA. Measurement of the absorption at 280 nm (A_{280}) provides information about purity if the quotient $Q_{na} = \frac{A_{260}}{A_{280}}$ is calculated. Pure DNA exhibits a quotient of about $Q_{na} = 1.8$ and pure RNA a quotient of about $Q_{na} = 2.0$. Contamination with protein or aromatic substances decreases the quotient due to their absorption at 280 nm.

6.6.3 Synthesis of complementary DNA

For the detection of mRNA transcripts via polymerase chain reaction (PCR) the reverse transcription of RNA into complementary DNA (cDNA) is a prerequisite. The first strand synthesis is catalysed by a viral reverse transcriptase, which is genetically engineered to be RNase H deficient. This enzyme requires oligodeoxynucleotides to prime the synthesis of single stranded DNA complementary to an RNA template. Two kinds of primers may be used. Polydeoxythymidine anneals to polyadenosine tails of mRNA molecules, resulting in a mRNA specific cDNA species. Random hexamers, which are used throughout the template have statistical sequences and therefore give a cDNA representing total RNA. RNA in RNA:DNA duplexes is subsequently degraded by RNase H.

Solutions used

PCR buffer (10×)

Tris	2.42	g	200	mM
potassium chloride	3.73	g	500	mM
hydrochloric acid	ad pH 8.4			
water, double-distilled	ad 100	ml		

The buffer is sterile filtered and stored aliquoted at -20 °C. This buffer and the following solutions are constituents of the 'SuperScript preamplification system' from Life Technologies (Eggenstein, D).

Reaction mix		final concentration
PCR buffer (10×)	4 µl	
25 mM magnesium chloride	4 µl	2.5 mM
dNTP mix (10 mM each)	2 µl	0.5 mM each
0.1 M 1,4-dithiothreitol	4 µl	10 mM

For denaturation of RNA, 2 µg of total RNA and 200 ng random hexanucleotides in a total volume of 24 µl RNase-free water are incubated for 10 min at 70 °C. The solution is instantly put on ice and chilled for a minimum of 1 min. To the solution 14 µl of reaction mix are added, mixed gently and incubated for 5 min at room temperature. Then 2 µl of 200 U/µl SuperScript reverse transcriptase are added, mixed gently and incubated for another 10 min at room temperature and subsequently for 50 min at 42 °C. The products are denatured for 15 min at 70 °C. For the removal of complementary RNA 1 µl of 2 U/µl RNase H are added on ice and incubated for 20 min at 37 °C. Finally, the product is diluted with 359 µl of double-distilled water to give 400 µl of cDNA and stored at -20 °C.

6.6.4 Polymerase chain reaction

This powerful and sensitive technique for directly amplifying short segments of the genome was invented by Karry Mullis (SAIKI *et al.*, 1985). It requires the knowledge of the sequence on either side of the target region. Double-stranded DNA is denatured and the resulting single-stranded DNA annealed to short oligonucleotides. These oligonucleotides, primers, are used by a heat-stable DNA polymerase to prime synthesis of a complementary strand of the template DNA. This cycle can be repeated by denaturing the products, annealing the primers and elongation. Thus a region framed by two primers is amplified. The number of copies of the target is doubled with every cycle and therefore growing exponentially. A typical PCR reaction of 30 cycles theoretically amplifies the template $2^{30} = 1 \cdot 10^9$ fold. The reverse transcription of RNA into cDNA allows detection of gene expression using PCR (see chapter 6.6.3). After amplification the reaction products are separated on an agarose gel (see chapter 6.6.5).

All PCR reactions are carried out in a final volume of 25 µl in a GeneAmp PCR System 2400 thermocycler (Perkin Elmer, Weiterstadt, D) in 0.2 ml test tubes (Roth, Karlsruhe, D). Cross contamination is avoided by pipetting all components except of the template in a separate room. The template is then added using pipette tips with aerosol filters with separate pipettes.

Pipetting scheme for one assay

water, double-distilled	11.1	µl		
PCR buffer (10×)	2.5	µl		
50 mM magnesium chloride	0.75	µl	1.5	mM
20 µM primer	2.5	µl	2.0	µM
20 µM primer	2.5	µl	2.0	µM
dNTP mix (10 mM each)	0.50	µl	0.2	mM each
5 U/µl Taq polymerase	0.125	µl	10	mM
cDNA	5	µl		

For every pair of primers one assay containing water instead of template is included in each PCR experiment to check for contamination.

A typical PCR programme is shown below.

Initial denaturation	30 sec at 94 °C	Prolong to up to 2.5 min for genomic DNA
Amplification	12 to 36 cycles	
Denaturation	30 sec at 94 °C	
Annealing	30 sec at 55 °C	Temperature varies with the primers used
Elongation	30 sec at 72 °C	Prolong for amplicons longer than 500 bp
Final Elongation	9.5 min at 72 °C	Finishes started elongations
Chilling	4 °C	Stops the reaction

Every sample is supplied with 3 µl of DNA sample buffer (10×) and 10 µl are subjected to agarose gel electrophoresis (see chapter 6.6.5).

6.6.5 Agarose gel electrophoresis

Nucleic acids move inside an electric field towards the cathode due to their phosphate backbone. Agarose forms a porous matrix that allows large nucleic acid molecules to be separated. Double-stranded DNA is readily stained by planar aromatic cations such as ethidium bromide. This dye binds by intercalation which strongly enhances its fluorescence. Single-stranded DNA and RNA also stimulate the fluorescence of ethidium but to a lesser extend than does double-stranded DNA. The size of unknown nucleic acid molecules is determined by running markers of known size in parallel.

Solutions used

TBE buffer (10×)			final concentration	
Tris	108	g	89	mM
boric acid	55	g	89	mM
0.5 M EDTA, pH 8.0	40	ml	2	mM
water, double-distilled	ad 1000	ml		

DNA sample buffer (10×)

Ficoll 400	2.5	ml	25	%
2.5 % bromophenol blue	200	µl	0.72	mM
TBE buffer (10×)	500	µl		
water, double-distilled	ad 10	ml		

To 1.5 g of agarose 67.5 ml double-distilled water are added and brought into solution by boiling in a microwave oven at 600 W. The solution is refilled to 67.5 ml and supplied with 7.5 ml of TBE buffer (10×). When the temperature is below 80 °C, 3.75 µl of 10 mg/ml ethidium bromide are added and when cooled below 60 °C the solution is poured into a prepared tray. After setting, the comb is removed and the gel in the tray is installed horizontally into the electrophoresis chamber. The chamber is filled with TBE buffer so that the gel is completely covered. The sample supplied with one ninth of its volume with DNA sample buffer (10×) is loaded into the wells. The electrophoresis is performed at 100 V and 150 mA until the bromophenol blue has run two thirds of the gel length. Gels stained with ethidium bromide are photographed and digitalised on an UV-transilluminator using a CCD camera (Polaroid, Offenbach, D).

6.7 Cell biology

6.7.1 Preparation of pervanadate

Equal volumes of 20 mM hydrogen peroxide and 20 mM sodium vanadate are mixed and incubated for 15 min at room temperature. To destroy superfluous hydrogen peroxide, 1/100 volume of 20 mg/ml catalase is added. The 10 mM pervanadate solution is used within 10 min.

6.7.2 Measurement of proliferation

Proliferation can simply be described as increase of cell number. Thus, the estimation of cell number is a straightforward method to quantify proliferation. Adherently growing cells detach from the ground upon death and can be washed off. GILLIES *et al.* (1986) reported the following method based on this fact. After washing off dead cells, adherent cells are fixed using glutardialdehyde and subsequently stained with crystal violet. The bound dye can be released using Triton X-100 and this solution is measured in an ELISA photometer.

Solutions used

Fixing solution

50 % glutardialdehyde	250	µl	0.1	M
PBS	12.25	ml		

The solution has to be prepared freshly.

Crystal violet stock solution

crystal violet	15	mg	2	mM
PBS	15	ml		

The solution is sterile filtered stable for two months.

Dye solution

crystal violet stock solution	1.3	ml	0.2	mM
PBS	11.7	ml		

The solution has to be prepared freshly.

Destaining solution

Triton X-100	10	µl	0.2	%
PBS	5	ml		

Cells are grown and treated in 24-well plates. After treatment, the supernatant is aspirated and the cells washed with 250 µl of PBS per well. Each well is supplemented with 500 µl of fixing solution and incubated for 30 min at room temperature under moderate shaking. Hereafter, the fixing solution is aspirated and the wells are washed with 500 µl of PBS. Staining is achieved by adding 500 µl of dye solution per well and incubation for 30 min at room temperature under moderate shaking. Unbound dye is washed away by incubation in deionised water for 15 min. Subsequently, the water is poured off and the plate tapped dry on tissue paper. To release bound dye, 200 µl of destaining solution per well are added and incubated for ≥ 1 h at room temperature under moderate shaking. Of the solution, 100 µl are transferred into a microtitre plate and the absorption at 570 nm is measured using an ELISA photometer. Absorption values of control cells are set to 100 % and cell numbers of treated cells are calculated as percent of control.

6.7.3 Determination of cytotoxicity

Unspecific cell death, necrosis, is accompanied by swelling and finally lysis of the cell. Therefore, the cellular content is released *in vitro* into the cell culture supernatant, or *in vivo* into the surrounding tissue. To assess cytotoxicity, uptake or exclusion of dyes such as trypan blue, eosin and propidium iodide can be measured. Another method is the measurement of the

activity of enzymes like alkaline phosphatase or lactate dehydrogenase (LDH) which are inactivated or released upon necrosis, respectively.

With the used "cytotoxicity detection kit (LDH)" from Roche Diagnostics (Mannheim, D), LDH activity in the cell culture supernatant is determined in an enzymatic two-step reaction. In the first step, LDH catalyses the oxidation of lactate to pyruvate and reduces NAD^+ to $NADH + H^+$. In the second step, diaphorase transfers the hydrogen from $NADH + H^+$ to the yellow-coloured tetrazolium salt 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyl-4H-tetrazolium chloride. The product of the reduction, the respective red-coloured formazan salt is measured at 490 nm in an ELISA photometer.

Solutions used

Catalyst solution and dye solution are ready-to-use solutions

Reaction buffer

catalyst solution	2	µl
dye solution	90	µl

Cells are grown and treated in 24-well plates. After treatment, the cell culture supernatant is removed and clarified by centrifugation at 300 ×g for 5 min. Then, 50 µl of the supernatant are transferred into a microtitre plate and 50 µl of reaction buffer are added. The samples are incubated protected from light for 5 min to 30 min at room temperature. The absorption at 490 nm is measured using an ELISA photometer. Absorption values of control cells are set to 100 % and the rate of LDH release from treated cells is calculated as percent of control.

6.7.4 Detection of apoptosis

The process of apoptosis can be measured by using various methods. One of the most reliable methods is the measurement of DNA-fragmentation, which is an irreversible step of apoptosis. An endonuclease, which is activated at the end of the apoptosis signalling pathway cleaves nuclear DNA at accessible sites. The resulting nucleosomes or oligonucleosomes with multiples of 200 base pairs of DNA are released into the cytoplasm.

For the determination of cytoplasmic histone-associated DNA-fragments the commercially available kit "Cell death detection ELISA^{PLUS}" from Roche Diagnostics (Mannheim, D) is used. The test is performed on a streptavidin-coated microtitre plate. After addition of cell lysates a mixture of anti-histone biotin-antibodies and peroxidase-coupled anti-DNA antibodies is added. The anti-histone antibodies recognise the protein portion of the nucleosome and

immobilise it via streptavidin-biotin on the microtitre plate. The DNA coiled around the histones is recognised by the peroxidase-coupled anti-DNA antibodies. A chromogenic peroxidase substrate is added and measured with an ELISA photometer.

Solutions used

Lysis buffer, incubation buffer and substrate solution are ready-to-use solutions

Immunoreagent solution

anti-histone biotin-antibodies	4	µl
peroxidase-coupled anti-DNA antibodies	4	µl
incubation buffer	72	µl

Cells are grown and treated in 24-well plates. After incubation with the distinct apoptosis stimulus, the plate is centrifuged at 150 ×g for 5 min. The supernatant is removed, 200 µl of lysis buffer per well are added and incubated for 30 min at room temperature under moderate shaking. A clear cytosolic supernatant is prepared by centrifugation at 150 ×g for 10 min. Thereafter, 20 µl of the respective supernatant are added to a streptavidin-coated microtitre plate. After addition of 80 µl immunoreagent solution the probes are incubated for 2 h at room temperature under moderate shaking. Subsequently, the wells are washed three times with incubation buffer. The wells are supplied with 100 µl substrate solution and incubated for 10 min at room temperature in the dark. The absorption at 405 nm is measured using an ELISA photometer. Absorption values of control cells are set to 100 % and the apoptosis rates of treated cells are calculated as percent of control.