2. MATERIALS & METHODS

2.1 MATERIALS

2.1.1 General chemicals and materials

7-AAD (7-Aminoactinomycine D)	Molecular Probes
Acrylamide 30%/Bisacrylamide 0.8%	Roth
Adhesion slide	Biorad
Ammoniumpersulfat (APS)	Biorad
Ampholine pH 3.5-9.5	Pharmacia
Ampholine pH 6-8	Pharmacia
Ampholine pH 3.5-10	Pharmacia
Bovine serum albumin (BSA)	Sigma
Bromophenol blue	Merck
Coomassie brilliant blue R	Serva
Cytochalasin B	Sigma
Dimethylsulfoxid (DMSO)	J.T. Baker Chemicals
Dithiothreitol (DTT)	Sigma
Dry milk	Gl cksklee
Ethanol	Riedel de Haen
Ethylendinitrotetraaceticacid (EDTA)	Sigma
Formaldehyde 37%	Merck
Glycerol	Roth
Glycine	Merck
HEPES	Gibco
Isobutanol	Merck
Isopropanol	Riedel de Haen
Ionophore (A23187)	Sigma

β-Mercaptoethanol	Merck
Methanol	J.T. Baker Chemicals
Molecular weight marker	Gibco BRL
Mowiol (4-88)	Calbiochem
Nitrocellulose membrane	Sartorius
Paraformaldehyde	Sigma
Phorbolmyristacetate (PMA)	Sigma
Phosphate buffered saline (PBS)	Seromed
Phytohemagglutinine (PHA)	Welcome Diagnostics, Dartford, UK
Propidium iodide (PI)	Sigma
Serva Blue R	Serva
Sodiumdodecylsulphate (SDS)	Merck
Sodiumazid	Riedel de Haen
Sodiumcitrate	Merck
Sodiumvanadate	Merck
Sodiumflouride	Merck
TEMED	Biorad
3,3«,5,5«-Tetramethylbenzidine (TMB)	Sigma
Tris-Base	Merck
Triton X-100	Sigma
Trypan blue	Serva
Tween 20	Gerbu
Urea	Gibco BRL
3MM Whatman paper	Whatman (Maidstone, UK)
X-lay film (Biomax MR)	Kodak

All other chemicals used in this study were obtained in analytical grade from Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) or Sigma (M nchen).

2.1.2 Cell culture materials

Ficoll-Hypaque	Pharmacia, Uppsala, Sweden
Fetal bovine serum (FCS)	Sigma
L-glutamine 200 mM	Life Technology
Penicillin-Streptomycine (10000 IU/ml)	Gibco BRL
RPMI 1640-medium	Fisher Scientific
Sheep red blood cell suspension	ICN Biomedicals Corp.
Heparin Thrombophob-2500	Nordmark Arzneimittel (Uetersen)
Neubauer counting chamber	Brand (Wertheim)
Recombinant IL-2 (250000 U/ml)	Biotest (Dreieich)

Plastic cell culture materials were obtained from Greiner (N rtingen) and Nunc Int. (Denmark).

2.1.3 Inhibitors

Wortmannin	Sigma
KB 8301	PharMingen Int.
Protease inhibitor (cocktail tablets)	Boehringer Mannheim

2.1.4 Radiochemicals

^{3}H-thymidine	Amersham

2.1.5 Peptides

MAS:	CDYKDDDDKMASGVAVSDGVIK	(MW 2329.8 Da)
DAI:	CDYKDDDDKDAIKKKL	(MW 1914 Da)
WAP:	CDYKDDDDKWAPESAPLKSKM	(MW 2443 Da)
WAPQ:	CDYKDDDDKWAPESAPLQSQM	(MW 2441.8 Da)

Activated Penetratin I	Appligene (Illkirch, France)	(MW 2557.6 Da)

2.1.6 Technical equipment

Autoclave	Webeco
Autoradiography cassette	Suprema
Beta-Counter *LS1701*	Beckman
Blot apparatus *2117 Multiphor II semi-dry*	Pharmacia/LKB
CO_2-Incubator	Heraeus
Centrifuge:	
Megafuge 3.0 RS	Heraeus
Minifuge T	Heraeus
Ultracentrifuge L-70	Beckman
Eppendorfcentrifuge *5415 C*	Eppendorf
Flow cytometer *FACS Calibur*	Becton Dickinson
Film developer *Hyperprocessor*	Amersham
Gelelectrophoresis *Protean II Cell*	Biorad
Microscope:	
confocal laser scanning microscope	Leica
phase contrast microscope	Zeiss

Vortex MS1	IKA
pH-meter 240	Corning
Power supply:	
23023 Multidrive XL	Pharmacia/LKB
Pipettes	
Pipet boy	TecNoMara
Multiseppipette plus	Eppendorf
P1000, P200, P20, P10	Gilson
Shaker *Vibrax VXR*	IKA
Thermomixer 5436	Eppendorf

2.1.7 Antibodies

primary antibodies

antibodies	species/isotype	source/reference
anti-CD2 (M1)	mouse monoclonal IgG1	Meuer et al., 1984
anti-CD2 (M2)	mouse monoclonal IgG1	Meuer et al., 1984
anti-CD2 (3PT)	mouse monoclonal IgG1	S.F. Schlossman, Dana Faber Cancer Institute, Boston, USA
anti-CD3 (BMA030)	mouse monoclonal IgG1	R. Kurrle, Behringwerke, Marburg, Germany
anti-CD28 (CD28.05)	mouse monoclonal IgG1	PharMingen, San Diego, USA
anti-CD95 (Apo1)	mouse monoclonal IgG3	P. Krammer, German Cancer Research Center, Heidelberg, Germany
anti-CD95 (Apo1)	mouse monoclonal IgG3 (F(ab) fragments)	P. Krammer, German Cancer Research Center, Heidelberg, Germany
anti-PIP2 (KT10)	mouse monoclonal IgG2b	T. Takenawa, Institute of Medical Science, Tokyo, Japan

anti-FLAG (M2)	mouse monoclonal IgG1	Sigma, St. Louise, USA
anti-CD25-PE	mouse monoclonal IgG1	Becton Dickinson, Belgium
anti-HLA-DR-PE	mouse monoclonal IgG1	Becton Dickinson, Belgium
anti-CD69-PE	mouse monoclonal IgG1	Becton Dickinson, Belgium
anti-cofilin (AS24)	polyclonal rabbit serum	Samstag et al., 1994
anti-actin	polyclonal rabbit serum	Sigma, St. Louise, USA
anti-p85	polyclonal rabbit serum	Santa Cruz Biotechnology
anti-Rac1	polyclonal rabbit serum	Santa Cruz Biotechnology
anti-RhoA	polyclonal rabbit serum	Santa Cruz Biotechnology
anti-Cdc42	polyclonal rabbit serum	Santa Cruz Biotechnology
anti-CD95 ligand (C-20)	polyclonal rabbit serum	Santa Cruz Biotechnology

Secondary reagents

Goat anti-mouse IgG	DAKO
Goat anti-mouse IgG, PE-conjugated	Dianova
Goat anti-mouse IgG, Texas red-conjugated	Dianova
Goat anti-mouse IgG, horseradish peroxidase-conjugated	Dianova
Goat anti-mouse IgG2b, FITC-conjugated	Southern Biotechnology
Goat anti-rabbit IgG, PE-conjugated	Dianova
Goat anti-rabbit IgG, horseradish peroxidase-conjugated	Dianova
Donkey anti-rabbit IgG, Cy2-conjugated	Dianova
horseradish-peroxidase conjugated streptavidin	PharMingen
Texas-red conjugated streptavidin	Dianova

2.1.8 Cells

Human peripheral blood lymphocytes (PBLs) were isolated by Ficoll-Hypaque density centrifugation of heparinized blood from healthy donors. Monocytes were removed by plastic adherence of PBLs for 1h at 37¡C at a density of $2x10^6$ cells/ml. After depletion of plastic-adherent cells, PBLs were separated into T and non-T cells by rosetting with sheep red blood cells which bind to CD2 molecules on the surface of human T cells. The PBLs were incubated in a 1% sheep red blood cell suspension (ICN Biomedicals Corp.) for 45 min at room temperature. This was followed by Ficoll-Hypaque density gradient centrifugation and the B lymphocytes were removed from the interphase of the gradient. The pellet consisted of T cells complexed with sheep erythrocytes. Human peripheral blood T lymphocytes (PBTs) were obtained by treating the pellet with hypotonic ACK buffer (see below) for 5 min, which leads to lysis of the sheep red blood cells. After being washed, T lymphocytes were cultured in RPMI 1640 standard medium. (RPMI 1640 (Fisher Scientific) standard culture medium supplemented with 100 U/ml of penicillin/streptomycin, 4 mM L-glutamine (Life Technologies Inc.), 2% (v/v) HEPES (1M) and 10% (v/v) fetal bovine serum (Sigma)).

The human T cell clone D798.18 (TCR αβ/CD3+; CD2+; CD4+; CD8-) was kindly provided by Dr. S. Wesselborg. Clones were maintained in RPMI 1640 standard medium containing recombinant IL-2 (rIL-2, 25 U/ml) and restimulated with a feeder cell mixture every 14 to 20 days. The feeder cell mixture for 2×10^6 D798.18 cells consisted of 2×10^6 irradiated (30 Gy) PBLs plus 2×10^5 irradiated (90 Gy) SKW 6.4 cells (EBV-transformed lymphoblastoid cell line)/well in 24-well plates, and was further supplemented with PHA (0.5 g/ml) and rIL-2 (25 U/ml).

The human CD4+ T lymphoma cell line Jurkat (JMP15G8) was cultured in RPMI 1640 standard medium.

ACK-lysis buffer:	0.15	M	NH_4Cl
	1.0	mM	$KHCO_3$
	0.1	mM	EDTA

2.2 METHODS

2.2.1 Stimulation of cells

For the induction of dephosphorylation of cofilin and its association with the actin cytoskeleton, PBTs were stimulated with mAbs. For CD2 triggering, PBTs were incubated with a mixture of mAbs directed against three different epitopes of CD2 (M1, M2, 3PT) in soluble form (5 g/ml each) for 15 min up to 8 hours as indicated in the figure legends at 37¡C. In some experiments PBTs were activated via CD3 and CD28 using immobilized mAbs. Culture wells were first coated with goat anti-mouse IgG (10 µg/ml, DAKO) at 4 ¡C over night, followed by binding of anti-CD3 (1 g/ml) and anti-CD28 (10 g/ml) mAbs for 2 hours at 37 ¡C. Alternatively, PBTs were stimulated with 10 nM PMA (Sigma) alone or plus different concentrations of calcium ionophore A23187 (0.5 - 1 M) for 10 min. For inhibition of PI3-kinase activity *in vivo*, cells were preincubated with 100 nM wortmannin (Sigma) for 20 min before cell stimulation. For the competitive inhibition of the interaction between cofilin and F-actin *in vivo*, PBTs were preincubated with different concentrations (1-5 M) of the cofilin derived peptides MAS or WAP alone, a mixure of both peptides, or equimolar amounts of the control peptide WAPQ, respectively, for 2 hours at 37¡C. Subsequently cells were stimulated and the actin cytoskeletal fractions were isolated as described below.

For T cell proliferation, 2.5 x 10^5 PBTs were stimulated with a mixture of CD2-specific mAbs (M1,M2, 3PT) in soluble form at different concentrations (0.25-1 g/ml, each) for

48 hours at 37 ¡C. Alternatively, PBTs were stimulated with various concentrations of PMA (1-10 nM) and 0.5 M calcium ionophore (A23187) for 48 hours. For some experiments culture supernatants were collected 48 hours after CD2 stimulation and further used for the quantitation of IL-2 and γIFN contents by ELISA. For the optimal expression of activation markers on the surface of PBTs, cells were stimulated with CD2 mAbs (M1, M2, 3PT; 1 g/ml each) for 16 hours for CD69 induction, for 24 hours for CD25 induction and for 48 hours for the upregulation MHC class II (HLA-DR) expression, respectively.
Peptide inhibition assays were performed by preincubation with the combination of MAS and WAP (2.5 M each) or the control peptide WAPQ (5 M) for 2 hours before cell stimulation. Stimulations of PBTs were performed in RPMI 1640 standard medium in all assays .

For the induction of apoptosis, the T cell clone D798.18 was washed with IL-2 free medium and then starved for 4 hours in medium without IL-2. Subsequently the cells were stimulated with soluble anti-CD2 antibodies (M1, M2, 3PT, 2.5 g/ml each). Optimal induction of apoptosis was obtained 6 hours after CD2 stimulation for Annexin V assays, and after 8 hours of stimulation for TUNEL assays. Alternatively, apoptosis was induced in D798.18 via CD95 triggering with 1 g/ml mAb anti-CD95 (Apo 1) for 4 hours. In some experiments the CD95/CD95 ligand interaction was blocked by incubating the cells with soluble anti-CD95 F(ab) fragments at a concentration of 0.5 - 1 g/ml.
For the induction of the expression of CD95 ligand, the D798.18 T cell clone was stimulated with CD2 antibodies (M1, M2, 3PT, 2.5 g/ml each) for 5-6 hours in IL-2 free standard medium. For inhibiting the cleavage of membrane bound CD95 ligand, the cells were preincubated with 10 M of the metalloprotease inhibitor KB 8301 (PharMingen) for 30 min at 37 ¡C before CD2 stimulation.
For the inhibition of the interaction between cofilin and F-actin *in vivo*, D798.18 cells were preincubated with a mixture of MAS and WAP peptides (2.5 M, each) or equimolar amounts of the control peptide WAPQ (5 M) for 2 hours at 37¡C before CD2 stimulation.

2.2.2 Preparation of cytoplasmic and actin cytoskeletal fractions

PBT cells (1 x 10^8 cells) or Jurkat cells (2 x 10^7) were washed twice in ice-cold PBS and resuspended in 1 ml hypotonic buffer containing 42 mM KCl, 10 mM Hepes pH 7.4, 5 mM $MgCl_2$, 5 mM iodoacetamide, 1mM Na_3VO_4, 20 mM NaF and protease inhibitors (cocktail tablets Complate™, Boehringer Mannheim). After an incubation for 30 min at 4¡C, the suspension was passed ten times through a 25-Gauge needle, then centrifuged at 300 x *g* for 5 min to pellet nuclei. The supernatant was collected and 5 mM EDTA and 0.5% TX-100 were added. After a 30 min incubation on ice, detergent lysates were centrifuged at 10000 x g for 1 hour. The detergent soluble supernatant which contains the cytoplasmic components was recovered. The detergent insoluble pellet, which contains cytoskeletal proteins, was washed once with lysis buffer by centrifugation at 10000 x *g* for 15 min. Cytoskeletal actin and actin-binding proteins were released from this detergent insoluble pellet by treatment with cytochalasin B (Sigma, St. Louis, USA) at a final concentration of 0.1 mg/ml diluted in 60 l lysis buffer for 1 hour at room temperature on a shaker. Afer centrifugation at 140000 x *g* for 45 min, the supernatant (ªactin cytoskeletal fractionª) was separated by 14% SDS-PAGE.

2.2.3 SDS-polyacrylamide gel-electrophoresis (SDS-PAGE)

Proteins were separated under denaturing conditions by discontinuous SDS-polyacrylamide gel-electrophoresis (SDS-PAGE) using the system of Laemmli (1970). In this discontinuous gel system the proteins are focused in a stacking gel into narrow bands. After leaving the stacking gel, the proteins enter the separating gel, which has a smaller pore size, a higher salt concentration and a higher pH compared to the stacking gel, and the denatured proteins are separated according to their molecular weight.

The discontiuous polyacrylamide gel consisted of a 14 % separating gel and a 4 % stacking gel and was prepared in a Biorad-Minigel Casting System. The protein samples were denatured by boiling for 5 min in Laemmli reducing sample buffer before loading onto the gel. Electrophoresis was carried out at 20 mA per gel in 1 x SDS-running buffer.

4% stacking gel:	0.5 M Tris/HCl pH 6.8	1.0 ml
	30% acrylamide/bisacrylamide mix	0.58 ml
	H_20	2.3 .ml
	10% SDS	40.0 l
	10% ammoniumpersulfat (APS)	40.0 l
	TEMED	4.0 l
14% separating gel:	1.5 M Tris/HCl pH 8.6	3.0 ml
	30% acrylamide/bisacrylamide mix	5.6 ml
	H_20	3.4 ml
	10% SDS	120 l
	10% ammoniumpersulfat (APS)	60 l
	TEMED	6.0 l

Laemmli sample buffer:	62.5 mM	Tris/HCl, pH 6.8
	5% (v/v)	β-Mercaptoethanol
	2% (w/v)	SDS
	20% (v/v)	glycerol
	0.5 % (w/v)	bromphenol blue
Running buffer:	25 mM	Tris/HCl, pH 8.3
	192 mM	glycine
	0.1 % (w/v)	SDS

2.2.4 Non-equilibrium pH gradient electrophoresis (NEPHGE)

The one-dimensional non-equilibrium pH gradient electrophoresis (NEPHGE) is an adaptation of the method developed by O«Farrell (1975) for two-dimensional gel electrophoresis. This modified isolectric focusing allows the separation of proteins according to their specific isolectric points in urea-containing polyacrylamide slab gels. The movement of proteins through pores in the polyacrylamide gel matrix is modulated by a pH gradient created by soluble ampholytes. This method was used to detect the phosphorylation state of cofilin in cytoplasmic and actin cytoskeletal fractions.

The NEPHGE slab gel was prepared by pouring a 6% acrylamide solution supplemented with urea and ampholytes into gel-plate sandwiches of the Mini-Protean II Cell gel system (Bio-Rad). The urea was dissolved in the gel solution in a 37 ¡C water bath, before polymerization was induced by adding TEMED and APS. The protein samples were mixed with urea and 9x O«Farrell buffer in a ratio of 4:4:1 (v/w/v) and were dissolved at 37 ¡C. Then the samples were loaded onto the gel. The upper buffer chamber was filled with acidic H_3PO_4 and the lower buffer chamber with basic NaOH solution. The power supply was connected with the anode at the top (H_3PO_4-containign reservoir) and the kathode at the bottom (NaOH-containing reservoir). Electrophoresis was carried out for 30 min at 100 V, followed by 250 V for 4 hours.

Gel solution (for 1 gel):	30% acrylamide/bisacrylamide mix	2.0	ml
	Urea	5.5	g
	Ampholine pH 3.5-9.5	0.5	ml
	10% ammoniumpersulfat (APS)	40.0	l
	TEMED	4 .0	l
	H_2O	add 10	ml

9x O«Farrell lysis buffer:	1.17 M	DTT
	0.4% (w/v)	Ampholine pH 3.5-10
	1.6% (w/v)	Ampholine pH 6-8
Running buffer (upper reservoir):	0.085% (v/v)	H_3PO_4
Running buffer (lower reservoir):	20 mM	NaOH

2.2.5 Western-blotting

Following gel electrophoresis, the proteins were electroblotted onto nitrocellulose membranes (Sartorius, 0.2 m) and subsequently analyzed by immunoblotting (Western blotting). The transfer of the proteins was carried out in a semi-dry transfer apparatus (Pharmacia, LKB). The polyacrylamide gel, the membrane and 3MM papers were equilibrated in blotting buffer for 5 min. 3MM papers were placed on the anode and overlaid with the nitrocellulose membrane followed by the gel containing the proteins. This was covered with 3MM papers. The kathode was placed on top and the proteins were electrotransferred for 45 min at 37 mA per gel.

The membranes were blocked for 2 hours at room temperature in Tris-buffered saline (TBS) containing 5 % nonfat dry milk. Then the membranes were rinsed three times with washing buffer (TBST) and were incubated overnight at 4¡C with rabbit antisera directed against cofilin (2 g/ml) or actin (2 g/ml) diluted in TBST/5% nonfat dry milk. After three washes in TBST, membranes were incubated for 1 hour with horseradish-peroxidase conjugated goat anti-rabbit IgG (1:10.000; Dianova). Following three 10 min washes in TBST, the blots were developed with an enhanced chemiluminescence system (ECL-kit, Amersham, Les VIis, France) and exposed to an Kodak BiomaxMR film for 30 seconds to 10 min depending on the strength of the signal.

Blotting buffer:	39 mM	glycine
	48 mM	Tris-base
	0.001% (w/v)	SDS
	20% (v/v)	methanol
Washing buffer (TBST):	10 mM	Tris, pH 7.5
	150 mM	NaCl
	0.1 % (v/v)	Tween-20

2.2.6 Coupling of peptides to Penetratin and purification of the conjugates

Synthetic peptides were produced by an automatic synthesizer (ABI 433) according to the Fmoc strategy (the sequences are shown in 2.1.5). The cysteine residue at the N-terminus of the peptides was used for coupling to Penetratin, a 16 amino acid region of the *Antennapedia* homeodomain, by means of a disulfide bond. Equimolar amounts of Activated Penetratin I (Appligene) and oligopeptide were incubated for 2 hours at room temperature. The coupling was carried out in water at a final concentration of 1 mg/ml.

The coupled peptides were purified by preparative HPLC (Shimadzu, LC-10AD) on an YMC-Pack ODS. S-5 mm 120A reverse phase column (20 x 150 mm) using an eluent of 0.1 % trifluoroacetic acid and 80 % acetonitrile. The fractions corresponding to the purified protein were lyophilised. The purified material was characterized by analytical HPLC and laser desorption mass spectrometry Vision 2000 (Finnigan MAT).

2.2.7 Enzyme-linked immunosorbent assay (ELISA)

Immunoprecipitation was performed using a modification of the solid-phase immunoisolation technique (Tamura, et al., 1984). In a sandwich ELISA system, actin was immunoprecipitated from actin cytoskeletal fractions and co-immunoprecipitated cofilin was detected with cofilin specific antiserum. To this end, 96-well plates were coated with 5 g/ml anti-actin polyclonal rabbit serum (Sigma) overnight at 4¡C. Uncoated wells were used as a negative control. After three washes in TBST, actin cytoskeletal fractions corresponding to 1 x 10^6 PBTs or, alternatively, 2 x 10^5 Jurkat cells were added at 200 l per well and incubated overnight at 4¡C. After three washes in TBST, the non-specific binding sites were blocked with an excess of irrelevant protein by adding 200 l per well of bovine serum albumin (2% w/v) in washing buffer (TBST) for 2 hours at room temperature. For the detection of co-immunoprecipitated cofilin, the plate was incubated with a biotinylated cofilin specific antiserum (2 g/ml in TBST) for 2 hours at room temperature. After washing horseradish-peroxidase conjugated streptavidin (1: 20000 diluted in TBST) was added for 1 hour. After each incubation step the plate was washed five times with 150 l TBST. 100 l TMB solution (ready-to-use, Sigma) was used as substrate. The enzymatic reaction was stopped by the addition of 4N H_2SO_4 and absorbance was measured at 450 nm. Assays were run in triplicates.

For the *in vitro* peptide binding assays, actin was immunoprecipitated from actin cytoskeletal fractions of resting PBTs and binding of Penetratin coupled peptides was detected with a FLAG-specific mAb in a sandwich ELISA system. Following immunoprecipitation of cytoskeletal actin as described above, the plate was blocked with 2% (w/v) BSA in TBST. After washing, the plate was incubated overnight at 4 ¡C with 10 g/ml of the different Penetratin-coupled peptides (DAI, WAP, MAS, WAPQ) or uncoupled Penetratin. For the detection of peptide binding 1 g/ml FLAG specific mAb (M2) was added for 2 hours at room temperature, followed by incubation with horseradish-peroxidase conjugated goat-anti-mouse IgG (1: 5000; Dianova). After each

incubation step the plate was washed five times with 200 l TBST. 100 l TMB solution was used as a substrate and the enzymatic reaction was stopped by the addition of 4N H_2SO_4. Absorbance was measured at 450 nm. Assays were run in triplicates.

IL-2 and γIFN in T cell culture supernatants were quantitatively determined by a sandwich ELISA system, using commercially available immunoassay kits (Immunotech S. A.) according to the manufacturer«s instructions. PBT cells (2×10^5) were stimulated as described under 2.2.1 in 96-well plates and culture supernatants were collected and stored at -20¡C.

Microtiter plates precoated with mAb against human IL-2 or γIFN, respectively, were incubated overnight with 50 l of culture supernatant per well. After washing the plates with wash solution, 50 l of biotinylated detection antibody (anti-IL-2 or anti-γIFN) was added at the recommended dilution. Then 100 l of strepavidin-horseradish-peroxidase solution was added and the plates were incubated for 1 hour at room temperature. After 3 washes, plates were incubated with 100 l TMB-substrate solution per well. After 30 min the enzymatic colour reaction was stopped by the addition of 50 l of stop solution (4N H_2SO_4) and absorbance was measured at 450 nm. Concentrations were expressed in pg/ml as determined using the respective recombinant cytokines as standards.

TBST:	10 mM	Tris, pH 7.5
	150 mM	NaCl
	0.1 % (v/v)	Tween-20

2.2.8 CD2 receptor cap formation and immunofluorescence microscopy

For the induction of receptor cap formation 5 x 10^5 purified PBT cells were incubated for 15 min at 37¡C with the CD2 antibody 3PT in soluble form (15 g/ml) in 50 l RPMI without FCS. Then the cells were washed and primary antibodies were cross-linked using Texas-red-labeled goat-anti-mouse IgG (Dianova) at 20 g/ml in RPMI without FCS at 37 ¡C for 45 min. Receptor capping was stopped by three washes in ice-cold PBS/0.2 % sodium azide and the cells were transferred onto adhesion slides (Bio-Rad Laboratories, Richmond, CA) and incubated for 15 min in PBS/0.2 % sodium azide. Slides were rinsed once with PBS and the non-specific binding sites were blocked by adding 30 l per well of bovine serum albumin (2% w/v) in PBS for 20 min at room temperature. The cells were fixed and permeabilized with methanol for 3 min at -20¡C and in acetone for 5 sec at 4 ¡C followed by three washes in PBS. For counterstaining of cofilin, actin, PI3-kinase, Rac1, RhoA, or Cdc42, cells were incubated with the appropriate rabbit antiserum for 2 hours at 4 g/ml in PBS/0.5%BSA. Following three rinses with PBS, the cells were incubated for 1 hour with Cy2-labeled donkey-anti-rabbit IgG (Dianova) at a 1:200 dilution in PBS/0.5% BSA.

Alternatively, caps were formed with biotinylated CD2 antibody cross-linked with streptavidin, which was used for demonstrating the co-localization of PtdIns(4,5)P_2 (PIP_2) with CD2 caps. To this end 2 x 10^5 cells were incubated with a biotinylated CD2 antibody for 15 min (3PT, 5 g/ml) in 20 l RPMI without FCS on adhesion slides at room temperature. After washing 3x in PBS, this was followed by an incubation with Texas-red-labeled streptavidin (1:500, Dianova) for 45 min at room temperature. Slides were rinced again and subsequently, cells were fixed with 4% paraformaldehyde (w/v in PBS) at room temperature for 30 min. Then the cells were incubated with the PIP_2 specific mAb KT10 (1:100) followed by an incubation with FITC-conjugated anti-mouse IgG_{2b} (1:100, Southern Biotechnology).

After washing 3 x in PBS and 1 x in ddH_2O the slides were mounted in Elvanol. Digitized images were generated using a confocal laser scanning microscope (TCS NT, Leica, Germany). For the evaluation of two-color experiments digital images were overlayed electronically.

Elvanol:	Mowiol (4-88)	5.0 g
	PBS, pH 8.0	20.0 ml
	glycerol	10.0 ml

2.2.9 Surface and intracellular staining for flow cytometry

Flow cytometry was used for analyzing the expression of cell surface and intracellular molecules on a single cell level. Data were acquired and analyzed on a FACS-Calibur (Becton-Dickinson) using the Cell-Quest software (Becton-Dickinson).

The expression of surface activation markers on PBTs was detected by immunostaining with directly labeled antibodies. PBTs were stimulated as described under 2.2.1. $5x\ 10^5$ cells were washed once with 200 l RPMI 1640 standard medium and stained directly with 50 l PE-conjugated anti-CD69, anti-CD25 or anti-HLA-DR (100 test kits, Beckton-Dickinson) diluted 1:10 in standard medium for 30 min on ice. As a negative control, cells were stained with an isotype matched immunoglobulin of irrelevant specificity at the same concentration (IgG_1, Becton-Dickinson). After washing the expression of surface activation markers was analyzed by flow cytometry.

The expression of CD95 ligand on the surface of the T cell clone D798.18 was detected by indirect immunostaining. D798.18 cells were stimulated as described under 2.2.1 in the presence or absence of the metalloprotease inhibitor KB 8301. Cells were washed twice

with standard medium and were then incubated with rabbit anti-serum against CD95 ligand (C-20) at a concentration of 20 g/ml for 30 min on ice. After three washes the cells were resuspended in secondary antibody solution (PE-conjugated donkey-anti-rabbit immunogloublin, diluted 1:100 in standard medium) and incubated for 30 min on ice. As a negative control, cells were stained with secondary antibody only. Cells were washed three times and the expression of CD95 ligand was analyzed by flow cytometry.

The internalization of Penetratin coupled peptides into PBTs was determined by intracellular flow cytometric analysis. Freshly isolated PBT cells (1×10^6) were incubated with 2.5 M Penetratin coupled peptides in 100 l RPMI standard medium for 2 hours at 37¡C. As a negative control, T cells were placed in standard culture medium only. Cells were washed once with PBS and were then fixed for 30 min with 4% paraformaldehyde. After fixation, cells were permeablized with 0.1% Triton X-100 for 10 min. Cells were washed 1 x with PBS and followed by an incubation with anti-FLAG mAb (10 g/ml) for 30 min in PBS containing 0.5% BSA. After three washes, the cells were incubated for 30 min with PE-conjugated goat-anti-mouse IgG (Dianova), which was diluted 1:100 in PBS/0.5% BSA. All incubations were carried out on ice. Cells were washed three times and the entered peptides were detected by flow cytometric analysis.

2.2.10 Proliferation assay

Cell proliferation was analyzed by measurement of DNA synthesis. To this end radioactive [^{3}H]-thymidine was added to the cells and its incorporation into DNA was quantified.

PBTs were seeded into 96-well tissue culture plates (2.5×10^5/well) and stimulated as described under 2.2.1. The cultures were incubated at 37¡C for 48 hours. Then the cells were pulsed with [^{3}H]-thymidine (1 Ci/well) and cultures were maintained for an

additional 16 hours. After this incubation period the cells were harvested by vacuum aspiration onto glass fiber filters. While free [^{3}H]-thymidine was washed through the filters, the [^{3}H]-thymidine incorporated in the DNA was retained. The radioactivity retained on the filters was measured by liquid scintillation counting. All assays were run in triplicates.

2.2.11 Determination of apoptosis

The induction of apoptosis in the humanT cell clone D798.18 was detected by Annexin V and TUNEL assays.

Annexin V-FITC was used to quantitatively determine cells undergoing early apoptosis. It relies on the property of cells to loose membrane asymmetry in the early phase of apoptosis, thereby the membrane phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane. In the presence of Ca^{2+}, Annexin V has a high affinity for PS. Therefore, staining with Annexin V-FITC in combination with vital dyes such as propidium iodide (PI) or 7-amino-actinomycin D (7-AAD) allows to identify early apoptotic cells (Annexin-V positive, PI or 7-AAD negative). Cells that stain positive for both Annexin V-FITC and PI or 7-AAD are either in the later stages of apoptosis, are undergoing necrosis, or are already dead before stimulation.

After induction of apoptosis as described in 2.2.1, D798.18 cells were washed twice with ice-cold PBS. Cells were resuspended in binding buffer and then transferred into a V-bottomed 96-well plate (5 x 10^5 cells in 100 l per well). Cells were incubated for 15 min at room temperature in the dark with 50 l FITC-conjugated Annexin V reagent (R&D Systems), which has been diluted 1:50 with binding buffer. Then cells were washed once with binding buffer and were resuspended in 100 l PI or 7-AAD at a final concentration of 2 g/ml each in binding buffer. After 5 min incubation 400 l binding buffer was added and the samples were analyzed without any further washing by flow cytometry. Annexin V-

FITC was detected by the FL1 PMT, PI was detected by the FL2 PMT and 7-AAD by the FL3 PMT. Appropriate electronic compensation was required to exclude any emission spectra overlap. The following controls were used to set up compensation and quadrants: unstained cells, cells stained with Annexin V-FITC only, cells stained with PI or 7-AAD

Binding buffer:	10 mM	HEPES/NaOH, pH 7.4
	140 mM	NaCl
	2.5 mM	$CaCl_2$

One of the later steps in apoptosis is DNA fragmentation, which was detected by TUNEL (terminal deoxynucleotidyltransferase dUTP nick end labeling) assays with an *in situ* cell death detection kit (Boehringer Mannheim). Cleavage of genomic DNA during apoptosis yields double-stranded, low molecular weight DNA fragments as well as single strand breaks in high molecular weight DNA. Those DNA strand breaks can be identified by labeling free 3«-OH termini with modified nucleotides in an enzymatic reaction using terminal deoxynucleotidyl transferase (TdT), which catalyzes polymerization of nucleotides to free 3«-OH DNA ends in a template-independent manner. Fluorescein labels incorporated into nucleotide polymers are detected and quantitated by flow cytometry.
D798.18 cells were washed twice with ice-cold PBS and transfered into a V-bottomed 96 well plates (1x 10^6 cells in 100 l per well). Cells were fixed with 100 l 4% paraformaldehyde in PBS, pH 7.4 for 30 min at room temperature. Then cells were washed once with PBS and resuspended in 100 l permeabilisation solution (see below) for 2 min on ice. After being washed twice with PBS, cells were incubated with 50 l TUNEL reaction mixture (Boehringer Mannheim, see below) for 60 min at 37¡C in the dark. After three washes, the cells were analyzed by flow cytometry. Cells in label solution (see below) without TdT were used as a negative control.

Permeabilisation solution: 0.1% (v/v) Triton X-100
0.1% (w/v) sodium citrate
(in PBS, pH 7.4)

TUNEL reaction mixture: 450 l Label solution (containing FITC-labeled dUTP)
50 l Enzyme solution (containing TdT)
(*in situ* cell death detection kit; Boehringer Mannheim)