2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General chemicals and materials

7-AAD (7-Aminoactinomycin D)	Molecular Probes
Acrylamide/bisacrylamide mix (30%T, 2.67% C)	Roth
Adhesion slide	Biorad
Ammoniumpersulfate	Sigma
Bovine serum albumin (BSA)	Serva
Brij-58	Pierce
Bromphenol blue	Merck
Cholera toxin B subunit (biotinylated)	Sigma
Cytochalasin B	Sigma
2-Deoxyglucose	Sigma
Dexamethasone	Sigma
Dimethylsulfoxid (DMSO)	Merck
Ethanol	Riedel de Haen
Ethylendinitrotetraaceticacid (EDTA)	Sigma
Fetal bovine serum (FCS)	Sigma
Ficoll-Hypaque	Pharmacia
Formaldehyde 37%	Merck
Glycerol	Roth
Glycine	Roth
HEPES	Sigma
Isopropanol	Riedel de Haen
Ionophore (A23187)	Sigma
Labtek chamber slides	Nunc Int.
β-Mercaptoethanol	Sigma
Methanol	Riedel de Haen
3MM Whatman paper	Whatman
Modified RPMI 1640 (without methionine, cysteine)	Sigma
Molecular weight marker (full range; rainbow marker)	Amersham Buchler
Mowiol (4-88)	Calbiochem
Nonidet P-40 (NP-40)	Sigma

Nylon wool fiber	Polysciences Europe
Paraformaldehyde	Sigma
Penicillin-streptomycin	Gibco
Phorbolmyristacetate (PMA)	Sigma
Plastic cell culture material	Greiner
Phalloidin-TRITC	Sigma
Poly-L-lysine	Sigma
Polystyrene latex beads (6 m fl)	Polyscience
Polyvinyliden-difluorid (PVDF)-membrane	Millipore
Propidium iodide	Sigma
Protein G Sepharose	Pharmacia
Protein A Sepharose	Pharmacia
Proteinase K	Merck
RPMI 1640	Bio Whittaker
Saponin	Sigma
Sodium orthovanadate	Merck
Sodium fluoride	Sigma
TEMED (N,N,N',N'-Tetramethylethylendiamin)	Sigma
TMB (3,3',5,5'-Tetramethylbenzidine)	Sigma
Triton X-100	Sigma
Trypan blue	Serva
Tween 20	Serva

All other chemicals used in this study were obtained in analytical grade from Merck, Roth, Serva or Sigma.

2.1.2 Inhibitors

Nocodazole (microtubule inhibitor)	Calbiochem
PP2 (inhibitor of src family protein tyrosine kinases)	Calbiochem
Complete™ protease inhibitor cocktail tablets	Boehringer Mannheim
Wortmannin (inhibitor of phosphatidylinositol 3-kinase)	Sigma
LY 294002 (inhibitor of phosphatidylinositol 3-kinase)	Calbiochem
Ocadaic acid (inhibitor of serine phosphatases type 1 and 2)	Research Biochemicals Int.

2.1.3 Radiochemicals

All radiochemicals were purchased from Amersham Buchler (Braunschweig)

[^{32}P]-γATP
adenosine 5'-[γ-^{32}P]triphosphate, triethylammonium salt
> 185 TBq/mmol (> 5000 Ci/mmol)
370 MBq/ml (10 mCi/ml)

Pro-mix L-[^{35}S] in vitro cell labeling mix
amino acid mixture containing L-[^{35}S]methionine and L-[^{35}S]cysteine
> 37 TBq/mmol (> 1000 Ci/mmol)
530 MBq/ml (14.3 mCi/ml)

[^{3}H]-thymidine
0.74-1.1 TBq/mmol (20-30 Ci/mmol)
37 MBq/ml (1 mCi/ml)

2.1.4 Expression vectors

Eucaryotic expression vectors encoding for myc-epitope tagged dominant negative mutant forms of rhoA (myc-rhoN19) and rac1 (myc-racN17), respectively, were kindly provided by Mark Simons (ONYX)

2.1.5 Peptides

The synthetic peptide HA 317-329, covering the C-terminal of the HA1 subunit of the human influenca virus A/PR/34/8 (H1N1), was produced by Dr. R. Pipkorn (DKFZ-Heidelberg) with an automatic synthesizer (ABI 433) according to the Fmoc strategy.

amino acid sequence (HA 317-329): **VTGLRNIPSIQSR**

2.1.6 Antibodies

primary antibodies

antibodies	species/isotype	source/reference
anti-mouse-CD3 (17A2)	rat monoclonal IgG2a	MacDonald, Epalinges, CH
anti-mouse-CD3 (145-2C11)	hamster monoclonal	ATCC CRL 1975
anti-mouse-CD44s (IM7)	rat monoclonal IgG_{2b}	ATCC TIB 235
anti-mouse-CD44s (KM81)	rat monoclonal IgG_{2a}	ATCC TIB 241
anti-mouse-CD44v10 (K926)	rat monoclonal IgG_{2a}	M. Roesel, DKFZ Heidelberg
anti-mouse-CD25-FITC (7D4)	rat monoclonal IgMκ	Southern Biotechnology
anti-mouse-CD69-FITC (H1.2.F3)	hamster monoclonal	Pharmingen
anti-mouse-CD95 (Jo-2)	hamster monoclonal	Pharmingen
anti-mouse-CD95-FITC (Jo2)	hamster monoclonal	Pharmingen
anti-mouse-CD95L-PE (MFL3)	hamster monoclonal	Pharmingen
anti-mouse- (331.12)	rat monoclonal IgG_{2b}	ATCC TIB 129
anti-human-c-myc (9E10)	mouse monoclonal IgG_1	ECACC 85102202
anti-mouse-IL-2 (JES6-1A12)	rat monoclonal IgG_{2a}	Pharmingen
anti-mouse-IL-2-biot. (JES6-5H4)	rat monoclonal IgG_{2b}	Pharmingen
anti-phosphotyrosine (PY99)	mouse monoclonal IgG_{2b}	Santa Cruz Biotechnology
anti-lck (3A5)	mouse monoclonal IgG_{2b}	Santa Cruz Biotechnology
anti-fyn (FYN3)	rabbit polyclonal	Santa Cruz Biotechnology
anti-CD3-ζ (6B10.2)	mouse monoclonal IgG_1	Santa Cruz Biotechnology
anti-ZAP-70 (LR)	rabbit polyclonal	Santa Cruz Biotechnology
anti-vav (C-14)	rabbit polyclonal	Santa Cruz Biotechnology
anti-PLCγ1 (1249)	rabbit polyclonal	Santa Cruz Biotechnology
anti-LAT (06-807-MN)	rabbit polyclonal	Upstate Biotechnology
anti-erk1 (C-16)	rabbit polyclonal	Santa Cruz Biotechnology
anti-phospho-erk (E-4)	mouse monoclonal IgG_{2a}	Santa Cruz Biotechnology
anti-c-jun (N)	rabbit polyclonal	Santa Cruz Biotechnology
anti-phospho-c-jun (KM-1)	mouse monoclonal IgG_1	Santa Cruz Biotechnology
anti-rac1 (C-14)	rabbit polyclonal	Santa Cruz Biotechnology
anti-rhoA (119)	rabbit polyclonal	Santa Cruz Biotechnology
anti-cdc42 (P1)	rabbit polyclonal	Santa Cruz Biotechnology

secondary reagents

goat-anti-rat IgG (whole molecule), FITC-conjugated	Southern Biotechnology
goat-anti-rat IgG_{2b}, FITC-conjugated	Bethyl Laboratories, Inc.
anti-hamster IgG, FITC-conjugated (absorbed against mouse and rat IgG)	Southern Biotechnology
sheep-anti-rat IgG, horseradish peroxidase conjugated	Amersham Buchler
donkey-anti-mouse IgG (minimal cross-reactivity), horseradish peroxidase-conjugated	Dianova
donkey-anti-mouse IgG (minimal cross-reactivity), Cy2-conjugated	Dianova
donkey-anti-rabbit IgG (minimal cross-reactivity) horseradish peroxidase-conjugated	Dianova
donkey-anti-rabbit IgG (minimal cross-reactivity) Texas red-conjugated	Dianova
streptavidin, horseradish peroxidase conjugated	Pharmingen

2.1.7 CD44 receptor globulin

Generation of the CD44-RG has been described by Zawadzki et al., 1998. CD44 cDNA was cloned into the pHT4 vector, which contains besides of regulatory elements the coding region of the κ-chain constant region (Traunecker et al., 1986). The CD44 cDNA coding for the extracellular part of the molecule (nucleotides 1-801) was amplified by PCR. The amplification product was ligated into the pT7T3 vector for sequencing. After digestion with SacI/HindIII the CD44 parts were ligated into the pHT4 expression vector. The myeloma line Ag8 was transfected by electroporation and selection medium (250 g/ml Xanthin, 8 g/ml mycophenolic acid) was added after 48 h.

2.1.8 Experimental animals

BALB/c mice were obtained from WIGA, Sulzfeld , Germany or were bred in the animal facilities of the German Cancer Research Center. Animals were kept under specific pathogen free conditions and were fed conventional diet and water ad libitum. Mice were used within 24h after birth or at the age of 6-8 weeks.

2.1.9 Cell lines

IP12-7 murine $CD4^+$ T cell hybridoma
specific for influenca virus hemagglutinin (HA317-329)
kindley provided by Dr. E. Rajnavolgyi (L. Ectovos University, God, Hungary)

2PK3 murine B-lymphoma line
(ATCC TIB 203)

IM7 mAb-IM7-producing hybridoma cell line
(ATCC TIB 235)

KM81 mAb-KM81-producing hybridoma cell line
(ATCC TIB 241)

K926 mAb-K926-producing hybridoma cell line
(M. Roesel, DKFZ Heidelberg)

145-2C11 mAb-145-2C11-producing hybridoma cell line
(ATCC)

Ag8 (P3X63Ag8.653) non-secreting murine myeloma cell line
(ECACC 85011401)

Ag8-CD44 receptor globulin (Ag8-CD44-RG)
transfected Ag8 cells producing CD44-RG
(V. Zawadzki, DKFZ Heidelberg)

All cell lines were cultured in RPMI-1640 standard culture medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin and 10 % (v/v) fetal bovine serum.

2.2 METHODS

2.2.1 Cell culture

Eucaryotic cells were grown in culture flasks in a humidified 37 ¡C, 5% CO_2 incubator. Cells were maintained in culture by feeding every 2 to 3 days with complete RPMI culture medium (RPMI 1640 supplemented with 10% (v/v) heat-inactivated FCS and 100 U/ml of penicillin/ streptomycin) until they reached confluency. Cells in suspension cultures were passaged in a ratio of 1:4 to 1:10. Adherent cells were dispersed by trypsin treatment (0.25% (w/v) trypsin in PBS) and reseeded into fresh cultures.
For long-term storage cells were frozen and stored in liquid nitrogen. Cell suspensions were washed with PBS and resuspended in freezing medium (10% (v/v) DMSO in cell culture medium). Cells were transferred into cryovials and the vials were placed for 1 hour to overnight in a -70 ¡C freezer, before transferring them to a liquid nitrogen storage freezer. Cryopreserved cells were thawed rapidly in a 37 ¡C water bath and were subsequently resuspended in prewarmed culture medium. Following one washing step to remove residual DMSO, the cells were plated at high density to optimize recovery.
Cell number and viability was determined with a hemacytometer and trypan blue staining. 1 volume of 0.4% trypan blue in 0.9% NaCl was mixed with 1 volume of cell suspension. The number of viable (unstained) cells was counted in an Improved Neubauer Hemacytometer and cell density was calculated.

PBS (pH7.2)	137 mM NaCl
	2.7 mM KCl
	4.3 mM Na_2HPO_4
	1.4 mM KH_2PO_4

2.2.2 Lymphocyte preparation and fractionation

Mice were killed by cervical dislocation. Thymus, spleen and lymph nodes were collected aseptically in PBS, were meshed through fine gauze and the resulting single cell suspensions were washed three times in PBS before resuspending them in RPMI 1640.

Macrophages were removed from spleen cell suspensions by plastic adhesion. After 1-2 hours incubation at 37 ¡C in plastic culture plates at a density of $3x10^6$ cells/ml, non-adherent cells were collected.
Erythrocytes were removed by Ficoll-Hypaque density centrifugation. Single cell suspensions were layered over Ficoll-Hypaque. After centrifugation for 15 min at 800 x g, the lymphocyte population was collected at the interface.
In some experiments T lymphocytes were enriched by nylon wool fiber separation. A 20 ml column packed with 10 ml volume of nylon wool was equilibrated with prewarmed (37 ¡C) complete RPMI 1640. Single cell suspensions ($1-2x10^8$ cells) were allowed to enter the column and after 1 hour of incubation at 37 ¡C the non-adherent T cells were collected by two washes with 15 ml RPMI 1640.
All cell preparations were washed three times in PBS and resuspended in RPMI 1640.

2.2.3 Stimulation of cells

Antigenic activation of the T cell line IP12-7 was measured by culturing $2x10^4$ IP12-7 cells with $2x10^4$ 2PK3 B lymphoma cells, prepulsed with different concentrations of HA 317-329 peptide in 96 well flat bottom tissue culture plates in complete RPMI. Cultures contained, in addition, 50 l supernatant of either the CD44-RG producing Ag8 transfectant or of the empty vector transfected Ag8 cells as a control. After 24 h of culture supernatants were harvested and IL-2 secretion was determined in a sandwich ELISA.

If not stated otherwise, all cross-linking studies were performed with immobilized antibodies. Plastic culture plates were coated with antibodies by incubating the plates at 4 ¡C overnight with the indicated antibodies adjusted to the appropriate concentration in 0,05 M Tris-HCl, pH 9.5. The plates were washed three times with PBS and blocked with 1% (w/v) BSA in PBS for 1 h at 37 ¡C. After three washes with PBS, the coated plates were used for cell stimulation.
For the induction of expression of cell surface activation markers and cytokine secretion, T lymphocytes were added to culture plates precoated with either anti-CD3, anti-CD44, control antibodies or combinations of anti-CD3 plus anti-CD44, respectively. Expression of CD69 was monitored after 12 h of culture by flow cytometric analysis. After 48 h of culture, CD25 surface expression was determined by flow cytometry and IL-2 production was estimated by a cytokine ELISA from culture supernatants.

CD95 and CD95L expression was induced by culturing IP12-7 cells for 16 hours on plates coated with anti-CD3, anti-CD44 or both anti-CD3 plus anti-CD44. At the end of the incubation time cells were harvested and surface expression of CD95 and CD95L was monitored by flow cytometry.

In the experiments on the induction of tyrosine phosphorylation, ERK1/2 activation and c-jun-phosphorylation, cells ($2.8x10^5$ T lymphocytes or $4x10^4$ IP12-7 cells) in 40 l RPMI 1640 were seeded into 96 well flat bottom culture plates, which have been precoated with either anti-CD3, anti-CD44, isotype matched control antibodies or combinations of anti-CD3 plus anti-CD44, respectively. Cells were allowed to incubate at 37 ¡C for the indicated times. Then, the cells were lysed by the addition of 20 l Laemmli reducing sample buffer and boiled immediately for 5 min. Whole cell lysates were resolved by SDS-PAGE and analyzed by Western-blotting using specific antibodies.

Cell stimulation for subsequent immunoprecipitation or sucrose gradient centrifugation was carried out in petridishes (100 mm in diameter) precoated with various antibodies. IP12-7 cells ($5x10^6$ cells/plate) or T lymphocytes ($2x10^7$ cells/plate) were added to the petridishes and incubated for the indicated times at 37 ¡C. The stimulation was terminated by transferring the dishes to ice and immediate lysis of the cells. The culture supernatants were gently removed and centrifuged, while adherent cells were lysed in the corresponding lysis buffer (see sections below). The lysate of the adherent cells was used for lysis of the cells pelleted from supernatants of the same culture. Lysis was carried out at 4 ¡C for 30 to 60 min under continuous shaking.

For the induction of morphological changes IP12-7 cells were plated on antibody-coated plastic dishes and were incubated at 37 ¡C for the indicated times. Cells were viewed with an inverse microscope (Leica), and phase-contrast images of the cells were generated. In some experiments cells were preincubated with pharmacological inhibitors or carrier DMSO for 20 min at 37 ¡C.

2.2.4 Antibody purification

Hybridoma supernatants were purified by passage over protein-G Sepharose 4B. To this end 1 l of hybridoma culture supernatant was passed over a protein-G column. The column was subsequently washed with 0.1 M phosphate buffer, pH 8.5. Afterwards bound IgG was eluted from the column with 0.1 M glycine buffer, pH 2.5. The eluted fractions were photometrically analyzed for proteins. Protein containing fractions were dialyzed against PBS, concentrated and filter-sterilized.

2.2.5 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.
Spleen cells (SC), lymph node cells (LNC), or purified T cells ($2x10^5$/well) were seeded in 96 well flat bottom plates, which had been precoated with either anti-CD3, anti-CD44, isotype matched control antibodies or combinations of anti-CD3 plus anti-CD44, respectively. In some experiments cells were stimulated with latex beads (6 m) precoated with antibodies. Polystyrene latex beads (Polyscience) were incubated under rotation for 90 min at room temperature with various concentrations of the indicated antibodies in PBS, followed by blocking with 1% BSA in PBS at 4¡ C overnight. Cells ($1.5x10^5$) were cultured with $7.5x10^4$ beads in 96 well round bottom microplates. [^{3}H]-thymidine (10 Ci/ml) was added after 48 hours and cultures were maintained for an additional 16 hours. After this incubation period the cells were harvested by vacuum 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 in a β-counter. All assays were run in triplicates.

2.2.6 Cytokine ELISA

Standard ELISA procedure was used (Engvall and Perlman, 1971). In a sandwich ELISA, 96 well plates (MaxiSorp; Nunc) were coated overnight with 5 g/ml anti-IL-2 capture antibody in binding buffer. Plates were washed and blocked with 1% BSA. Plates were incubated overnight with 50 l of culture supernatant. After washing the plates with PBST, biotinylated anti-IL-2 detection antibody (2.5 g/ml in PBST/BSA) was added. After incubation for 1 h at room temperature, the plates were washed and HRP-coupled streptavidin (1:10000 in PBST/BSA) was added. Plates were incubated for 30 min at room temperature. After each incubation step the plates were washed five times with PBST. TMB substrate solution (ready to use; Sigma) was added and after 30 min the enzymatic color reaction was stopped by the addition of 4N H_2SO_4. Absorbance was read at 450 nm (reference 550 nm). Concentrations were expressed in pg/ml as determined using recombinant IL-2 as standard. Assays were run in triplicates.

Binding buffer	0,05 M Tris-HCl, pH 9.5
PBS (pH7.2)	137 mM NaCl 2.7 mM KCl 4.3 mM Na_2HPO_4 1.4 mM KH_2PO_4
PBST	0,05 % (v/v) Tween 20 in PBS
PBST/BSA	1% (w/v) BSA in PBST

2.2.7 Immunofluorescence staining for flow cytometry

Flow cytometry was used for analyzing the expression of cell surface molecules on a single cell level. Data were acquired and anlyzed either by an EPICS-XL (Coulter, D sseldorf, Germany) or a FACS-Calibur (Becton Dickinson, Heidelberg, Germany).
The expression of surface activation markers, as well as the expression of CD95 and CD95L was detected by immunostaining with directly labeled antibodies. Cells were stimulated as described under 2.2.3. At the end of the stimulation, cells were harvested and washed with icecold PBS. 2-5x10^5 cells per sample were directly stained for 30 min on ice with saturating amounts of FITC- or PE-labeled antibody diluted in 50 l PBS/FCS (PBS supplemented with 2% (v/v) FCS). Samples were washed three times with icecold PBS and resuspended in PBS containing 1 g/ml 7-aminoactinomycin D (7-AAD). 7-AAD is an nucleic acid dye, which was used for the exclusion of nonviable cells in the flow cytometric assays, since it does not cross the plasma membrane of living cells. Fluorescence signals of living cells were determined by flow cytometry with appropriate electronic compensation to exclude any emission spectra overlap.
The expression of CD44 and CD3 on the surface of IP12-7 cells was determined by indirect immunostaining. 2-5x10^5 IP12-7 cells were washed with PBS and were incubated with 50 l of the primary antibody solution (1-5 g/ml in PBS/FCS) for 30 min on ice. After three washes in icecold PBS, the cells were resuspended in secondary antibody solution (fluorochrome-coupled secondary antibody in PBS/FCS at the concentration recommended by the supplier) and incubated for 30 min on ice. Cells were washed three times with PBS and resuspended in PBS containing 1 g/ml 7-AAD in order to discriminate live from dead cells.

2.2.8 Determination of apoptosis

Apoptosis was induced by culturing thymocytes of newborn mice (1 x 10^6 cells / ml) or the IP12-7 T_H line (2.5 x 10^5 cells / ml) for 20 hours on anti-CD3 and/or anti-CD44 coated 24 well plates. To block activation induced apoptosis, in selected experiments anti-CD95 mAb (clone Jo2) or isotype-matched hamster IgG was added to the cultures in soluble non-crosslinked form. Some of the cultures were established in the presence of dexamethasone (Sigma) at the indicated concentrations. Thereafter cells were harvested and programmed cell death was evaluated by the following methods: Annexin V-FITC / PI double staining, flow cytometric measurement of hypodiploid nuclei and DNA ladder assay.

For the evaluation of programmed cell death by annexin V-FITC / PI double staining an apoptosis detection kit (R&D Systems) was used. Early apoptotic cells with exposed phosphatidylserine bind annexin V-FITC but exclude PI, whereas necrotic or late apoptotic cells are both annexin V-FITC and PI positive. The cells were incubated with annexin V-FITC and PI at room temperature in 100 l binding buffer (10 mM HEPES, pH 7.4; 140 nM NaCl; 25 mM $CaCl_2$). After 15 min 400 l of binding buffer was added and flow cytometric analysis was performed with appropriate electronic compensation to exclude any overlap of the two emission spectra.

One of the later events in apoptosis is DNA fragmentation, which was detected by DNA ladder assay in agarose gels or by flow cytometric measurement of hypodiploid nuclei.

For the DNA ladder assay, cells were resuspended in 400 l DNA-lysis buffer (40 mM Tris-HCl, pH 7.5; 40 mM EDTA; 0,2% (w/v) SDS) containing 200 g/ml proteinase K and incubated for 2h at 56 ¡C. Samples was extracted once with 400 l phenol and twice with the same volume of phenol/chloroform/isoamylalcohol (25:24:1 (v/v/v)). DNA was recovered by centrifugation after overnight precipitation at -20 ¡C in two volumes of ethanol in the presence of 0,3 mol/l Na-acetate. Pellets were washed with icecold 70 % (v/v) ethanol, air-dried and dissolved in 50 l TE (10 mM Tris-HCl; 1 mM EDTA; pH 7.5). After addition of 1/10 gel-loading buffer (25% (w/v) Ficoll type 400; 0,25% (w/v) bromphenol blue; 0,25% (w/v) xylencyanol), the samples were run overnight at 30V on a 1% agarose gel containing 1 mg/ml ethidium bromide. DNA fragments were visualized under UV-light.

For the flow cytometric measurement of hypodiploid nuclei (Nicoletti et al., 1991), cells were centrifuged and the pellets were gently resuspended in hypotonic PI solution (PI 50 g/ml in 0.1% sodium citrate plus 0.1% Triton X-100). The tubes were then placed at 4¡ C in the dark overnight. The PI fluorescence of individual nuclei was measured by flow cytometry and the percentage of apoptotic nuclei (hypodiploid peak in the DNA fluorescence histogram) was calculated.

2.2.9 Biosynthetic labeling of cellular proteins

Biosynthetic labeling of cellular proteins was achieved by placing cells in a nutritional medium containing all components necessary for cell growth in culture, including radiolabeled proteins. To metabolically label cells with [^{35}S]-methionine and [^{35}S]-cysteine, cells were washed three times in PBS and were resuspended at 5x10^6 cell/ml in methionine/cysteine-free cell culture medium containing 10 % (v/v) dialyzed FCS (dialyzed against PBS to remove unlabeled amino acids that would decrease labeling efficiency). The cells were incubated for 30 min at 37 ¡C, followed by a 6-h incubation in the same medium containing 0.25 mCi/ml Pro-mix L-[^{35}S] cell labeling mix (amino acid mixture consisting of L-[^{35}S]-methionine and L-[^{35}S]-cysteine). After *in vitro* labeling, the cells were washed three times with PBS and were subsequently processed for immunoprecipitation.

2.2.10 Immunoprecipitation

For immunoprecipitation of CD44 from metabolically labeled cells, 5x10^6 radioactive labeled cells (v. 2.2.9) were lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 10 mM NaF; 1 mM Na_3VO_4; 1% (v/v) Brij-58). For the detection of tyrosine phosphorylation of PLCγ, vav and ZAP-70, cells were stimulated as described under 2.2.3 and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 10 mM NaF; 1 mM Na_3VO_4; 1% (v/v) Nonidet P-40; 0.25% (w/v) Na-deoxycholate). In some experiments fyn was immunoprecipitated from IP12-7 cells (5x10^6 cells/sample), which have been lysed in lysis buffer (20 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA; 10 mM NaF; 1 mM Na_3VO_4) containing either 1% (v/v) Triton X-100 or 1% (v/v) Triton X-100 plus 0.2% (w/v) saponin. All lysis buffers contained protease inhibitors (complete™ protease inhibitor cocktail tablets, Boehringer Mannheim). Cell lysis was carried out at 4 ¡C for 30 min under continuous shaking. After clarification by centrifugation for 10 min at 10000 x g cell lysates were subjected to immunoprecipitation.
For subcellular fractionation in the experiments on TCR ζ phosphorylation, stimulated cells (v. 2.2.3) were lysed for 30 min at 4 ¡C in TNE buffer (50 mM Tris-HCl, pH 8.0; 20 mM EDTA; 10 mM NaF; 1 mM Na_3VO_4; 1% (v/v) Nonidet P-40) containing complete™ protease inhibitors (Boehringer Mannheim). TNE lysates were centrifuged at 10000 x g for 10 min at 4 ¡C and the supernatant designated soluble fraction. The detergent insoluble pellet was resuspended in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0,1% (w/v) SDS, 1% (v/v) Nonidet P-40, 0.5% (w/v) Na-deoxycholate, 1 mM EDTA, 10 mM NaF; 1 mM Na_3VO_4, complete™

protease inhibitors. Insoluble proteins were extracted from the pellet by sonification, incubated for 30 min at 4 ¡C under vigorous shaking, and centrifuged at 10000 x g for 10 min at 4¡C. The supernatants were designated insoluble fraction.

For immunoprecipitation, lysates of $5x10^6$ IP12-7 cell or $2x10^7$ T lymphocytes were precleared by the addition of 5 g control antibody for 60 min followed by incubation with 1/10 volume Protein A Sepharose (Pharmacia) for 2 hours at 4¡ C. After centrifuging the sample for 1 min at 10000 x g to pellet the sepharose beads, the supernatant was used for specific immunoprecipitation. Precleared lysates were incubated for 60 min at 4 ¡C with 2 g of the indicated antibody. Protein A Sepharose (1:1 in PBS, $1/10^{th}$ of the total volume) was added for an additional 60 min. Anti-CD44 (IM7, ratIgG_{2b}) immunoprecipitations were performed with Protein G Sepharose instead of Protein A Sepharose, since ratIgG_{2b} binds only very weakly to Protein A. Immunoprecipitates were collected by centrifugation (1 min at 10000 x g) and the immune complexes were washed at least 4 times with the corresponding lysis buffer. Immunoprecipitates were analyzed by SDS-PAGE, followed by Western blotting and, in case of [^{35}S]-labeled proteins, autoradiography.

2.2.11 Immune complex kinase assay and reprecipitation

For *in vitro* kinase assay $2x10^7$ IP12-7 cells or $1x10^8$ thymocytes were lysed for 30 min at 4¡ C in 1 ml of IP-buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4) containing 1% Brij-58 and protease inhibitor cocktail (Boehringer Mannheim) and cleared by centrifugation (10000 x g, 4¡ C, 15 min). Lysates were precleared by the addition of 10 g control antibody for 60 min followed by incubation with 1/10 volume Protein G Sepharose for 2 hours (for immunoprecipitation see 2.2.10). Afterwards lysates corresponding to $1x10^7$ IP12-7 cells or $5x10^7$ thymocytes were incubated with anti-CD44 (IM7, 5 g/ml) or control antibody for 60 min at 4¡ C. Protein G Sepharose (1:1 in PBS, $1/10^{th}$ of the total volume) was added for an additional 60 min. Immune complexes were washed 4 times with IP-buffer 0.5% Brij-58 and 1 time with incomplete kinase buffer (100 mM NaCl, 20 mM HEPES, pH 7.4). The beads were resuspended in 20 l complete kinase buffer (100 mM NaCl, 20 mM HEPES, pH 7.4, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 1 M ATP) supplemented with 10 Ci [^{32}P]-γATP and *in vitro* phosphorylation was carried out for 15 min at 37¡ C. The reaction was stopped by the addition of 10 l Laemmli reducing sample buffer and boiling. Alternatively, 100 l 1.2% SDS was added for 30 min at room temperature. After dilution with immunoprecipitation buffer containing 0.1% Brij-58 and 1 mg/ml BSA, the *in vitro* phosphorylated proteins were reprecipitated with the indicated antibodies.

Immunoprecipitated proteins were analyzed by SDS-PAGE, followed by western blotting and autoradiography.

2.2.12 Purification of GEM fractions

GEM fractions were isolated due to their low buoyant density by sucrose gradient ultra-centrifugation. Cells were stimulated as described under 2.2.3, and were lysed for 30 min in 1 ml of ice-cold TNE-buffer (20 mM Tris-HCl, pH 7.4; 150 ml NaCl; 2 mM EDTA; 10 mM NaF; 1 mM Na_3VO_4) containing 0.5% Triton X-100 and complete protease inhibitor cocktail (Boehringer Mannheim). The lysate was adjusted to 40% (w/v) sucrose by mixing with 1 ml 80% (w/v) sucrose prepared in TNE-buffer. After transfer of the lysate to the centrifuge tube, 2 ml 30% (w/v) sucrose in TNE was carefully overlaid, then 1 ml 5% (w/v) sucrose in TNE was overlaid. After centrifugation of the samples for 16-18 hours at 200000 x g at 4 ¡C in a SW55Ti rotor (Beckman Instruments Inc.), 0.4 ml gradient fractions were collected from the top. GEMs were recovered mainly from the low-density fractions 2, 3, and 4. Fractions were analyzed by SDS-PAGE and Western blotting using specific antibodies. GEM fraction is pooled fractions 2-4 and the Triton X-100 soluble fraction is pooled fractions 10-12. GEM-associated fyn was solubilized in 1% Triton X100 plus 0.2% saponin in TNE-buffer at 4 ¡C before immunoprecipitation by anti-fyn mAb.

2.2.13 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 the 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 smaller pore size, higher salt concentration and a higher pH compared to the stacking gel, and the denatured proteins are separated according to their molecular weight.
Gel casting was performed in a BioRad Minigel Casting System. Proteins were resolved on separating gels containing from 7.5 to 12.5 % acrylamide. The stacking gel was prepared with an acrylamide concentration of 4 %. The protein samples were denatured by boiling for 5 min in reducing or non-reducing Laemmli sample buffer before loading onto the gel. Electrophoresis was carried out at 20 mA per gel in 1x SDS-running buffer.

Stacking gel	1.25 ml	0.5 M Tris-HCl, pH 6.8
	3.05 ml	H_2O
	50 l	10 % (w/v) SDS
	0.65 ml	Acrylamide/bisacrylamide mix (30% T, 2.67% C)
	25 l	10 % (w/v) ammoniumpersulfate (APS)
	5 l	TEMED

Separating gel	*% acrylamide*	*7,5*	*10*	*12.5*
	1.5 M Tris-HCl, pH 8.8	5.0 ml	5.0 ml	5.0 ml
	H_2O	9.7 ml	8.0 ml	6.3 ml
	10% (w/v) SDS	0.2 ml	0.2 ml	0.2 ml
	Acrylamide/bisacrylamide mix (30% T, 2.67% C)	5.0 ml	6.7 ml	8.4 ml
	10% (w/v) ammoniumpersulfate (APS)	0.1 ml	0.1 ml	0.1 ml
	TEMED	10 l	10 l	10 l

Laemmli sample buffer (4x)	*reducing*	*non-reducing*
Tris-HCl, pH 6.8	62.5 mM	62.5 mM
glycerol	20 % (v/v)	20 % (v/v)
SDS	2 % (w/v)	2 % (w/v)
β-mercaptoethanol	5 % (v/v)	

SDS-running buffer	25 mM Tris-base
	192 mM glycine
	0.1% (w/v) SDS

2.2.14 Western blotting

Following gel electrophoresis, the proteins were electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore) and subsequently analyzed by immunoblotting using specific antibodies. The transfer of the proteins was carried out in a "tank transfer system" (BioRad). The polyacrylamide gel, the foam pads and the 3MM papers were equilibrated in blotting buffer. A foam pad was placed on one side of the cassette holder and overlaid with a sheet of 3MM paper. The gel was placed on top of the 3MM paper and the PVDF-membrane, which had been prewetted in methanol for 15 seconds, was placed on top of the gel. This was covered by another sheet of 3MM paper and a foam pad was put on top. The cassette holder was closed and placed in the tank blotting apparatus with the membrane positioned on the anode side of the gel. The proteins were electrophoretically transferred in blotting buffer at 90 V for 1 hour or at 30 V overnight. Blots were blocked for 1 hour at room temperature in TWB containing 3% (w/v) BSA. The membranes were washed three times for

5 min with TWB and were incubated for 1 hour with primary antibodies diluted to the appropriate concentration (1-5 g/ml) in TWB. After three 10 min washes with TWB, membranes were incubated for 1 hour with horseradish-peroxidase conjugated secondary antibodies (1:10000 in TWB). At the end of the incubation time, the membranes were washed three times for 10 min in TWB. The blots were developed with an enhanced chemiluminescence system (ECL-kit, Amersham) and exposed to a X-ray film (Kodak) for 30 seconds to 10 min depending on the strength of signal. When the same blot was revealed with different probes, Ab-stripping was performed by incubating the membrane in stripping buffer at 50 ¡C for 30 minutes. The membrane was extensively washed in TWB and blocked by incubating for 1 hour in 3% (w/v) BSA in TWB. Subsequent immunodetection was performed as described above.

Blotting buffer	25 mM Tris-base 192 mM glycine 10 % (v/v) methanol
TWB (Tris washing buffer)	10 mM Tris-HCl, pH 7.4 150 mM NaCl 0.05 % (v/v) Tween 20
Stripping buffer	62,5 mM Tris-HCl, pH 6.7 100 mM 2-mercaptoethanol 2 % (w/v) SDS

2.2.15 Dot-blot immunoassay

Samples were dotted onto polyvinylidene fluoride (PVDF) filters (Immobilon-P, Millipore), using a Schleicher & Sch ll dot-blot apparatus. The PVDF membrane was prewetted for 15 seconds in methanol and was placed on top of a 3MM paper, which had been equilibrated in PBS. Sucrose gradient fractions were diluted in PBS and applied to the wells in a 200 l volume. The samples were dotted onto the membrane by gentle suction. After blocking the membranes for 60 min with TWB containing 3% (w/v) BSA, the dot-blotted filters were incubated with biotinylated cholera toxin B (5 g/ml) in TWB for 60 min at room temperature. Following three 10 min washes in TWB, the membranes were incubated for 60 min with horseradish-peroxidase-conjugated streptavidin at a 1:20000 dilution in TWB. All incubations

were carried out at room temperature. After thorough washing, the filters were developed with an enhanced chemiluminescence reagent (ECL-kit, Amersham) and exposed to a X-ray film (Kodak).

TWB (Tris washing buffer) 10 mM Tris-HCl, pH 7.4
150 mM NaCl
0.05 % (v/v) Tween 20

2.2.16 Immunofluorescence microscopy

CD44 receptor caps were induced by incubating $1x10^5$ IP12-7 cells for 15 min at 37 ¡C with the CD44 specific mAb IM7 in soluble form at 10 g/ml in 100 l RPMI containing 10% FCS. After washing the cells, primary antibodies were cross-linked using FITC-conjugated goat-anti-rat IgG_{2b} at 10 g/ml in RPMI supplemented with 10% FCS at 37 ¡C for 30 min. Receptor cap formation was stopped by two washes in ice-cold PBS. The cells were transferred onto adhesion slides (BioRad Laboratories) and were incubated for 15 min in PBS. After the cells had sedimented and anchored to the glass, slides were rinsed once with PBS. Cells were fixed for 30 min in 4% PFA (w/v in PBS).
Cell spreading was induced by layering IP12-7 cells ($1x10^5$ cells/ml in RPMI/10% FCS) over Labtek chamber slides (Nunc) which have been precoated with 10 g/ml anti-CD44 (IM7). As a control, cells were seeded onto chamber slides coated with poly-L-lysine ("ready to use" solution, Sigma). Cells were incubated at 37 ¡C for various times. At the end of the incubation period, wells were gently washed with PBS. Cells were fixed for 30 min in 4% PFA (w/v in PBS).

After fixation, slides were washed three times for 5 min in PBS. Cells were permeabilized by incubating them for 4 min in PBS containing 0.1% (v/v) Triton X-100. Slides were washed three times for 5 min with PBS, and non-specific binding sites were blocked by incubation in PBS/BSA for 20 min. After this blocking step, the cells were incubated with the primary antibody at an appropriate dilution (5-10 g/ml) in PBS/BSA for 60 min. Slides were rinsed three times in PBS, and subsequently, cells were incubated for 60 min with an appropriate fluorochrome-conjugated secondary antibody (diluted 1:100 to 2:200 in PBS/BSA). In some experiments cells were stained for F-actin by an additional 60 min incubation with phalloidin-TRITC (1 g/ml). Immunofluorescence staining was performed in a humidified chamber at

room temperature. After washing three times in PBS and once in H_2O, the slides were mounted in Elvanol. Digitized images were generated using a confocal laser scanning microscope (TS NT, Leica, Germany). For the evaluation of two-color experiments digital images were overlaid electronically.

PBS for immunofluorescence (pH 7.2)	NaCl	137 mM
	KCl	2.7 mM
	Na_2HPO_4	4.3 mM
	KH_2PO_4	1.4 mM
	$MgCl_2$	2 mM
4% PFA	4% (w/v) paraformaldehyde in PBS without Mg^{2+} (Goes into solution on heating to 65 ¡C for 1 h or on addition of NaOH. Store at -20 ¡C)	
PBS/BSA	PBS containing 0.5% (w/v) BSA	
Elvanol	Mowiol (4-88)	5.0 g
	PBS, pH 8.0	20 ml
	glycerol	10 ml

2.2.17 Transient transfection

Electroporation was used for transient transfection of IP12-7 cells with expression plasmids encoding myc epitope-tagged dominant negative mutant forms of rhoA or rac1, respectively. Aliquots of $5x10^6$ IP12-7 cells from exponentially growing cultures were resuspended in 400 l ice-cold serum-free RPMI 1640. 40 g of DNA was added to the cell suspension and the DNA/cell suspension was transferred into an 0.4 cm electroporation cuvette. Electroporation was performed by using an EUROGENTEC electroporation apparatus at a setting of 250 V and 900 F. Following transfection, the cells were incubated for 16 hours in RPMI containing 10% (v/v) FCS. Dead cells were removed by Ficoll-Hypaque density centrifugation. Cells were layered over Ficoll-Hypaque and after centrifugation for 15 min at 800 x g, the living cells were collected at the interface. The cells were washed three times with PBS and processed as indicated for each experiment.