

## **2. Material and methods**

### **2.1 Purification of primary human T cells**

The proliferative response of immortalized cell lines to growth factors and anchorage is variably dysregulated (Hughes and Pober, 1996; Rincon and Flavell, 1994). Therefore only primary human T lymphocytes were used in all experiments. The leukocyte-enriched fraction of donor blood obtained from the S. Raffaele hospital was used as a source of primary human T lymphocytes. Blood of healthy donors was centrifuged twice to separate erythrocytes and platelets from leukocytes at the hospital. T cell isolation was then carried out accordingly to a previously described protocol (Pardi et al., 1992). All steps were carried out sterilely. First, periphery blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque gradient centrifugation. Buffy coated blood was diluted with PBS (Phosphate Buffered Saline: 140mM NaCl, 2.7mM KCl, 1.8mM KH<sub>2</sub>PO<sub>4</sub>, 10mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2) and layered on a Ficoll cushion (Lymphoprep, Nycomed Pharma AS). The centrifugation was carried out at 800g without brake and low acceleration for 30 minutes in a swing-out rotor (Heraeus Minifuge RF) at room temperature. Cells were collected from the interphase and washed once in PBS at 800g for 15 minutes. Remaining erythrocytes were selectively lysed by incubating in 0.17M NH<sub>4</sub>Cl for 10 minutes at room temperature. Residual platelets were eliminated by centrifuging three times at 250g for 10 minutes. Monocytes were depleted by adherence to cell culture dishes in RPMI (GIBCO BRL, Life Technologies) supplemented with 2% foetal calf serum (FCS, HyClone Laboratories Inc.) for one hour at 37°C in a gassed incubator (Heraeus Instruments) at 5% CO<sub>2</sub>. Remaining monocytes and B cells were depleted by incubation for 45 minutes in a nylon wool column (Type 200L combed, scrubbed nylon wool, Robbins Scientific Corporation) which had been saturated for one hour with RPMI 10% FCS at 37°C. NK(natural killer) cells and residual antigen presenting cells were eliminated by a panning technique using anti-CD16 (KD1, IgG1) and anti-MHC II (CA141, IgG1) monoclonal antibodies (mAbs), respectively. The cells were incubated for 30 minutes at 4°C with 0.1µg/10<sup>6</sup> cells anti-MHC II and 0.3µg/10<sup>6</sup> cells anti-CD16 mAb and excess antibody was washed away with ice-cold PBS. Cells were then incubated for one hour at 4°C in RPMI 1% FCS in dishes, which had been pre-coated with polyclonal goat anti-mouse antibodies (Zymed). Dishes were pre-coated with 10µg/ml polyclonal goat anti-mouse antibodies in 50mM Tris (2-Amino-2-(hydroxymethyl)-1,3-propanediol, Boehringer Mannheim), pH 9.5 for 3 hours at room temperature, washed with PBS and blocked for one hour at room temperature with 1% BSA (bovine serum albumin, Sigma) in PBS. The separation was controlled by immunofluorescence (see 2.4) with anti-CD3, anti-CD56 (Bender MedSystems), anti-human Ig(Immunoglobulin, Dako immunoglobulins a/s) and anti-CD14 antibodies (gift of M. Alessio, Dibit)

as markers for T cells, NK cells, B cells and monocytes, respectively, using a Fluorescence Activated Cell Scanner (FacScan, Becton Dickinson & CO). Cells were usually 95-8% CD3-positive, contained no detectable monocytes, only traces of B cells and <<5% NK cells (not shown). Since resting B cells are very poor activators of T cells and NK cells do not present antigen, the residual non-T cells should not influence the activation process of the T cells

## **2.2 Stimulation of primary T cells**

### **a) Stimulation conditions**

Physiological stimulation of the TCR and LFA-1 occur in an intercellular contact of an antigen presenting cell and a T cell. In order to mimic the stimulation of the TCR/CD3 complex occurring in vivo, the mAb OKT3, which is specific for the CD3  $\epsilon$ -chain, was used in soluble or immobilized form. In order to stimulate LFA-1, either the anti- $\alpha$ L mAb TS1.22 or the purified, recombinant ligand of LFA-1, zz-ICAM-1 (Protein A-ICAM-1 fusion protein expressed in baculovirus-infected insect cells, gift of Anna Randi, Glaxo Welcome; for GST-ICAM expressed in *E. coli* see 2.6, 2.8 and 2.16) were used. CD28 was stimulated with soluble anti-CD28 mAbs (10U93, IgM) or with immobilised B7-1 or -2-Ig fusion proteins (secreted by myeloma cells, gift of Dr. P Dellabona, Dabit). Cells stimulated with soluble antibodies were first incubated on ice for 30 minutes with saturating concentrations of antibody ( $1\mu\text{g}/10^6$  cells), excess antibody was washed away with ice cold PBS and cells were resuspended in pre-warmed RPMI medium containing 10% FCS and  $10\mu\text{g}/\text{ml}$  goat anti-mouse Ig polyclonal antibody (Zymed). For stimulation with immobilized ligands, dishes were pre-coated with either 0.01% Poly-L-lysine (Sigma) in distilled water or  $10\mu\text{g}/\text{ml}$  antibodies or recombinant ICAM-1 in Tris-Cl 50mM pH 9.5 overnight at  $4^\circ\text{C}$  and subsequently blocked for 1 hour at room temperature in PBS, 1% FCS. Cells were plated in pre-warmed RPMI medium containing 10% FCS. In order to functionally block LFA-1, cells were stimulated in the presence of  $10\mu\text{g}/\text{ml}$  of the anti- $\alpha$ L mAb TS1.22 and the anti- $\beta$ 2 mAb TS1.18. In some cases, cells were stimulated in the presence of  $100\mu\text{g}/\text{ml}$  blocking anti-IL-2 antibodies (goat polyclonal, Sigma) or with  $50\text{ng}/\text{ml}$  PMA (Phorbol-12 Myristate-13 Acetate, Sigma) and  $0.5\mu\text{M}$  Ionomycin (a calcium-ionophore, Calbiochem); the latter is a well established positive control for both signal transduction and proliferation (Kumagai et al., 1987). Resting T cells do not adhere to ICAM-1 without prior activation (Dustin and Springer, 1989). In order to investigate events mediated by LFA-1 in the absence of TCR stimulation, two different approaches were used: The incubation in ICAM-coated dishes was either performed in the presence of  $1\text{mM}$  EGTA and  $5\text{mM}$   $\text{Mg}^{2+}$ , since this treatment induces the high affinity state of LFA-1 for its ligand (Stewart et al., 1996). Alternatively, the ICAM-1 fusion protein was substituted by the anti- $\alpha$ L

antibody TS1.22. The latter was exclusively used during long-term incubation, because the sequestration of  $\text{Ca}^{2+}$  blocks T cell activation (Kumagai et al., 1987).

### **b) Use of Inhibitors**

A major limitation in the manipulation of primary T cells is, that they can not be transfected with eukaryotic expression vectors without prior activation (Hughes and Pober, 1996). In order to obtain direct evidence for the importance of a signalling protein or pathway for proliferation, specific inhibitors were therefore used. For inhibition experiments cells were pre-treated with the respective inhibitor for 1 hour at 37°C. The Inhibitor of the polymerisation of the actin-based cytoskeleton, Cytochalasin D (Calbiochem), was used at 10 $\mu\text{M}$ . This relatively high concentration was needed to completely inhibit spreading induced with anti-CD3 or anti-LFA-1 antibodies (not shown). The immunosuppressant cyclosporin A (CsA) was used at 50-200ng/ml. It is believed to specifically inhibit IL-2 transcription by preventing the nuclear translocation of NF-AT, NF- $\kappa\text{B}$  and c-Rel. In fact, recombinant IL-2 nearly completely bypassed the CsA-mediated inhibition of proliferation of anti-CD3 stimulated cells, (not shown). The MKK (Map kinase kinase)-Inhibitor PD98059 (Calbiochem) was used at 50 $\mu\text{M}$ ; at this concentration it completely blocked ERK activation, but did not significantly inhibit the Map kinases JNK or p38 ((Gould et al., 1995), see Figure 3.6). The Inhibitor of the p38 Map kinase SB203580 (SmithKline Beecham) was used at 25 $\mu\text{M}$ . Herbimycin A inhibits members of the src-tyrosine kinase-family. It irreversibly binds to thiol groups of src-kinases and has been reported to lead to the partial degradation of p56<sup>lck</sup> (June et al., 1990). Cells were pre-treated for 16 hours with 1 $\mu\text{M}$  Herbimycin A, since this protocol has been reported to lead to a complete inhibition of p56<sup>lck</sup>, but only to 50% inhibition of its closed relative, p59<sup>lyn</sup> and to no significant inhibition of PKC-dependent events (June et al., 1990).

## **2.3 Propidium iodide staining**

Propidium iodide staining allows the quantification of cells in the G0/G1 (2n DNA content/cell) and the S/G2/M phases of the cell cycle (>2n-4n DNA content/cell) and was therefore used to quantify proliferation. After 3 days of stimulation cells were collected, washed with PBS and fixed for 30 minutes in 75% ethanol. Cells were then centrifuged and re-suspended in 0.1% sodium citrate containing 0.05% Nonidet(N)P-40 (Fulka Chemie AG), 50 $\mu\text{g/ml}$  propidium iodide (Sigma) and 50 $\mu\text{g/ml}$  RNaseA (which had been boiled in Tris pH 7.5, 15mM NaCl for 15 minutes to inactivate contaminating DNases; Sigma). Cells were incubated for at least 1 hour at room temperature and analysed by flow cytometry.

## **2.4 Immunofluorescence**

Staining with fluorochrom-labelled antibodies was performed to control the purity of the isolated T cells, to analyse the expression of the activation marker CD25 after stimulation (clone 3G10, FITC (Fluorescein isothiocyanate)-coupled, Boehringer-Ingelheim) and to assess TCR down-regulation. Incubation with both unlabelled and fluorochrome-conjugated antibodies were performed at  $1\mu\text{g}/10^6$  cells in PBS, 1% BSA (Sigma), 0.1% NaN<sub>3</sub> for 30 minutes at 4°C. Excess antibody was washed away with ice cold PBS. Analysis was performed by flow cytometry.

The downregulation of the TCR was assessed as follows: Cells were stimulated with immobilized or soluble, biotinylated anti-CD3 mAbs. Cells stimulated with soluble anti-CD3 mAbs were then harvested and stained with FITC-conjugated streptavidine. Cells stimulated with immobilized anti-CD3 mAb were further incubated for 30 minutes with biotinylated anti-CD3 mAb on ice after stimulation to saturate surface CD3 complexes, and then stained and analyzed as described.

## **2.5 F-actin staining**

In order to visualise the rearrangement of the actin based cytoskeleton fluorochrome-labelled Phalloidin, which inhibits the depolymerization of polymerized (F-)actin by binding to actin filaments, was used. Cells were incubated in pre-coated dishes, non adherent cells were washed away, cells were fixed in PBS 3.7% paraformaldehyde for 10 minutes at room temperature and washed three times with PBS. Cells were then permeabilized in PBS containing 0.05% NP-40 for 2 minutes on ice and washed as before. The incubation with the labelled phalloidin was performed at 200ng/ml in PBS, 1% FCS for 45 minutes at room temperature; excess phalloidin was washed away with PBS. The cells were photographed with a fluorescence microscope (Leitz, Dialux 20EB).

## **2.6 Cloning of the glutathione-S-transferase-intercellular adhesion molecule 1 fusion protein (GST-ICAM)**

The use of monoclonal antibodies as a substitute for the physiological ligand of surface receptors can result in artefacts (Nunes et al., 1994). Therefore one ligand of LFA-1, ICAM-1 was produced as a Glutathione-S-Transferase(GST)-fusion protein.

### **a) PCR amplification**

The complete coding region of human ICAM-1 containing plasmid (pCDM8, InVitrogen), which was available in the lab, was amplified with the Polymerase Chain Reaction (PCR) with the two primers 5'-

TCCCCCTCAGAATTCATCCTG-3' and 5'-CCCTTGCGGCCGCAGGTCCAG-3' (Gibco). The primers are homologue to the base pair triplets coding for the amino acids 30-37 (5'-3') and 188-194 (3'-5') and create recognition sites for EcoRI (GAATTC) due to an A-G and a G-T point mutation in primer 1 and for NotI due to an C-G point mutation in primer 2, respectively. The amplification product contains the coding region for the ligand binding site for LFA-1, which maps to the amino terminal immunoglobulin-superfamily domain 1 (amino acids 46-65). The Pfu polymerase was used for PCR, since this polymerase produces few errors due to its 3'-5' exonuclease proof-reading activity. In 100µl reaction volume 1µM of primer 1 and 2, 2.5 Units Pfu polymerase (Stratagene), 80ng ICAM-1-pCDM8 plasmid, 100µM dCTP, dATP, dTTP and dGTP (Pharmacia) and 10µl of 10xPfu-buffer (200mM Tris-Cl pH 8.0, 100mM KCl, 60mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20mM MgCl<sub>2</sub>, 1% TritonX-100, 0.1mg/ml nuclease-free BSA (Stratagene)) were mixed and PCR performed in a PCR thermocycler (GeneAMP PCR system 2400, Perkin & Elmer) as follows: The DNA was first denatured for 5 minutes at 94°C, then 30 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 59°C and two minutes elongation at 72°C were performed. After the last cycle additional 7 minutes of elongation were allowed. The reaction was controlled with an 1% TAE-(40mM Tris-acetate pH 7.8, 1mM ethylene-diaminetetraacetic acid (EDTA)) agarose gel and revealed that one single product of about 500 nucleotides as expected had been amplified.

#### **b) Purification of the ICAM(-DNA)-fragment**

The PCR product was mixed with an equal volume of a 1:1 mixture of chloroform and phenol, the sample was mixed vigorously for one minute and centrifuged for 5 minutes at room temperature in a table centrifuge (Heraeus) at 13000 rpm. The upper aqueous phase was recuperated and the salt concentration adjusted to 0.3M by addition of 3M sodium acetate, pH 5.2. Further, 2.5 volumes of absolute ethanol was added and the was DNA left at -80°C for 30 minutes. It was precipitated for 30 minutes at 13000 rpm in a table centrifuge (Heraeus, Biofuge 13) at 4°C, washed once with ethanol 70%, again centrifuged for 10 minutes at 13000 rpm and 4°C, and the pellet was resuspended in 25µl TE (Tris-Cl (10mM, pH 7.8) - EDTA (1mM)).

#### **c) Digestion with restriction enzymes**

Digestion of both fragment and plasmid with EcoRI and NotI creates sticky ends, which allow the insertion of the fragment into the plasmid's multiple cloning site *in frame*, resulting in the coding for the ICAM-1 fragment fused to GST under an inducible promoter. Both 1µg fragment and plasmid (pGEX-4T1 Promega) were digested separately in 30µl with 1 Unit of both EcoRI and NotI in 10mM Tris-Cl pH 8.5, 10mM MgCl<sub>2</sub> and 100mM KCl for 16 hours at 37°C. The control with an 1.2% TAE-agarose gel revealed that the plasmid had been completely linearized and that the fragment

remained a single band.(about 500 nucleotides long). The bands were excised from the gel and the DNA eluted with a kit (QIAEXII), which is based on DNA binding to silica beads at pH<7.5 and elution at pH>7.5. The purification was controlled on a 1.2% TAE-agarose gel.

#### **d) Ligation**

Two ligation reactions of 100ng linearised, purified plasmid and 3 or 5 fold excess of digested, purified fragment (about 30 and 50ng, respectively) were incubated for 16 hours at 16°C with 1U Ligase (Promega) in Ligase buffer (30mM Tris-Cl pH 7.8, 10mM MgCl<sub>2</sub>, 10µM DTT, 0.5mM ATP (Promega)) in a final volume of 20µl.

## **2.7 Competence induction and transformation of Escherichia coli**

#### **a) Competence induction**

E. coli was transformed with the obtained product of the ligation and ampicillin-resistant clones (hopefully due to ampicillin-resistance encoding plasmids) screened for GST-fusion protein expression. The E. coli strain BL-DR, which is both deficient for some proteases (possibly cleaving the expressed fusion protein) and for recombinase A (enhances plasmid mutations) was used for transformation and fusion protein expression. The bacteria have to be pre-treated in order to render them competent for plasmid DNA uptake: Bacteria from a single colony were inoculated in 3ml LB (Luria-Bertani Medium: 10g/l tryptone, 5g/l yeast extract, 10g/l NaCl) over night at 37°C under rotation. The inoculate was then diluted 1:100 in fresh LB and grown at 37°C until the absorbency at 550nm reached 0.48. The cells were then cooled down in ice and centrifuged for 10 minutes at 3000 rpm (Heraeus Minifuge RF) at 4°C. The pellet was resuspended in 40ml of solution 1 (30mM potassium acetate pH 5.8, 100mM KCl, 10mM CaCl<sub>2</sub>, 50mM MnCl<sub>2</sub>, 15% glycerole) and incubated for 5 minutes on ice. The cells were again pelleted at 3000 rpm for 5 minutes at 4°C and resuspended in 4ml solution 2 (10mM Pipes-KOH (free acid) pH 6.5, 75mM CaCl<sub>2</sub>, 10mM KCl, 15% glycerol). After a final incubation of 15 minutes on ice, the competent bacteria were aliquotated and stored at -80°C.

#### **b) Transformation**

For transformation, 100µl of competent bacteria were incubated for 30 minutes on ice with or without 10µl of the ligation reaction or with 20ng uncut plasmid as a positive control. The cells were heated for 1.5 minutes to 42°C, 900µl LB (37°C) was added and the bacteria were allowed to recuperate for one hour at 37°C. The plasmid encodes a resistance for ampicillin, on ampicillin-containing dishes only transformed bacteria should grow. Therefore 100µl of the bacteria were plated on ampicillin (50µg/ml) - LB-agar dishes and grown for 16 hours at 37°C.

## **2.8 Expression, purification and protease cleavage of GST fusion proteins**

Two GST fusion proteins were over-expressed in *E. coli* and purified: GST-c-Jun(1-79) for JNK-kinase assays (see 2.14), and GST-ICAM-1 for *in vitro* T cell stimulation.

### **a) Expression**

One colony was inoculated and grown for 16 hours at 37°C under agitation in 2x YTA (16g/l Tryptone, 10g/l Yeast extract, 5g/l NaCl, 100mg/l ampicillin). The culture was diluted 1:100 in fresh, pre-warmed 2x YTA and grown as before until the OD reached 1.0. The culture was then grown for one hour at room temperature and 100µM IsoPropyl-β-D-ThioGalactoside (IPTG) added to a final concentration of 0.1µM to induce the expression of the GST-fusion protein. The culture was grown for additional 4 hours under shaking at room temperature.

### **b) Purification**

Induced cells were centrifuged for 10 minutes at 4°C at 7700g (Heraeus, Minifuge) and resuspended in 50µl ice-cold PBS per ml of culture. Cells were disrupted by sonication (3 times 30 short strokes, Labsonic 2000U, Braun). TritonX-100 was added to a final concentration of 1% and samples were incubated for 30 minutes at 4°C. Cells were centrifuged for 10 minutes at 4°C and 12000g and the supernatant was saved and stored in aliquots at -80°C. In order to purify the expressed protein, to 1ml of the sonicate 20µl of in PBS equilibrated Glutathione Sepharose (4B, Pharmacia) was added and samples were incubated with gentle agitation for 30 minutes at room temperature. The suspension was centrifuged at 500g for 3 minutes and the pellet washed three times with PBS. For elution, bound proteins were dissociated from the beads by incubating three times in 100mM Tris-Cl pH 8.0, 20mM glutathione (Sigma), 120mM NaCl at room temperature for 10 minutes. Eluates were pooled and analysed by SDS-PAGE and subsequent Coomassie-staining (see 2.10) From eight colonies of bacteria transformed with GST-ICAM-1 tested, seven expressed a GST-fusion protein of about 45kD, in agreement with the predicted size and one expressed the original GST protein (not shown). The reactivity of the purified GST-ICAM-1 fusion protein with a blocking anti-ICAM-1 antibody was confirmed with an ELISA (see 2.16).

### **c) Protease cleavage**

In spite of the fact, that the GST-ICAM-1 fusion protein specifically reacted with a function-blocking anti-CD54 mAb in ELISA (2.16), it did not support LFA-1 mediated adhesion (not shown), probably due to the bulky aminoterminal GST. The fusion proteins was cloned into the pGEX4T1 plasmid, which encodes a thrombin cleavage site between GST and the multiple cloning site and the cloned protein can therefore be separated

from the GST part by a digestion with thrombin (Sigma): The fusion protein bound to the glutathione-beads were incubated with 50 cleaving Units thrombin/ml in PBS for 16 hours at 4°C. SDS-PAGE and Coomassie-staining revealed that GST-ICAM had been completely cleaved; the slower migrating band could be identified as the GST protein due to its molecular weight. The supernatant of the digestion contained no detectable ICAM-1 fragment. We concluded that it had been digested by thrombin and used the zz-ICAM (Protein A - ICAM-1 fusion protein) gifted from Glaxo Wellcome for T cell stimulation.

## **2.9 Purification of monoclonal antibodies**

Monoclonal antibodies were purified from hybridoma culture supernatant with a protein G affinity column by our technician Stella Putignano: Monoclonal antibodies were loaded on a protein G-column, which had been equilibrated with 20mM phosphate buffer pH 7.2. The column was then washed with the same buffer to wash away the contaminating proteins. Elution of the bound antibodies was achieved by washing the column with 100mM glycine, pH 2.7. The collected eluate was neutralized with NaOH and dialysed for 24 hours against PBS with at least three changes of the dialyzation buffer. Protein concentration was estimated by absorbency at 280nm and controlled by flow cytometry.

## **2.10 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), band shift assay, Coomassie-staining and immunoblotting**

Immunoblotting was performed to visualize phosphorylation events by band shift assay or phosphotyrosine-blotting or to monitor the expression levels of cell cycle regulated genes.

### **a) SDS-PAGE**

Prior to lysis, cells exposed to various stimuli were washed twice with ice cold PBS. Lysisbuffer (50mM Tris-Cl pH 7.4, 0.5% NP-40, 0.15M NaCl, 2mM EDTA, 10mM NaF, 10mM P207, 0.5mM Na<sub>3</sub>VO<sub>4</sub>, 100µg/ml PMSF, 1µg/ml Aprotinin and Leupeptin) was added and samples were incubated for 15 minutes on ice. The soluble fraction was cleared by centrifugation and protein concentration determined (see 2.11). Sample buffer was added to the samples, samples were boiled and equal amounts of protein loaded on a SDS-PAGE. The separating gel consisted of polymerized 6-15% acrylamide and 0.48-1.2% bisacrylamide, respectively, in 375mM Tris pH 8.8, 0.1% sodium dodecylsulfate (SDS) and 0.1% ammonium persulfate (APS). The stacking gel consisted of 5% acrylamide, 0.4% bisacrylamide, 125mM Tris pH 6.8, 0.1% SDS and APS. The gels were run in 25mM Tris, 192mM glycine, 0.1% SDS and the applied voltage was 5-15 Volts/cm.

### **b) Band-shift assay**

Post-translational modifications of proteins such as glycosylation or phosphorylation can result in altered migration in a SDS-PAGE and allows the quantification of Map kinase activation (Ferrell and Bhatt, 1997). Band shift assay were used as well to measure hyperphosphorylation of Raf-1 and the retinoblastoma protein (pRb). Optimal separation of hyperphosphorylated from hypo- or not phosphorylated proteins were achieved when the separating gel contained 10% acrylamide and 0.073% bisacrylamide.

### **c) Coomassie-staining**

Coomassie blue staining visualizes proteins separated in a SDS-PAGE: gels were incubated for 30 minutes in Coomassie Blue (0.25% Coomassie brilliant blue R-250 in 50% methanol and 10% acetic acid) and destained in 5% methanol and 7% acetic acid on a shaker at room temperature.

### **d) Immunoblotting**

The proteins separated in the SDS-PAGE were transferred on a nitrocellulose membrane (Amersham) in a transfer apparatus in transfer buffer (25mM Tris, 192mM glycine, 20% methanol). Blocking of non specific binding and all incubation with primary antibodies (0.1-5 $\mu$ g/ml) or horseradish peroxidase (HRP) coupled secondary antibody (1:4000; Zymed) were performed in blocking buffer (5% dry non-fat milk in Tris (50mM, pH 7.4) buffered saline, Tween20 0,05% (TBST)). The following primary antibodies were used: MAb anti-JNK1 (clone G151-333 Upstate), anti-cyclin D2 (G132-43, Pierce), anti-cyclin D3 (C-16, Santacruz), anti-c-Jun (KM-1, Santacruz), anti-ERK-2 (D-2, Santacruz), anti-fyn (15, Santacruz) anti-p16<sup>INK4</sup> (clone G175-405 Upstate), polyclonal rabbit anti-CDK2, 4 and 6, anti-pRb, anti-Raf-1, anti-lck, anti-p27<sup>kip1</sup>, anti-p38 and anti-cFos antibodies (Santacruz). In the case of phosphotyrosine-blotting, blocking and incubation with the biotinylated anti-phosphotyrosine mAb 4G10 (Upstate) and HRP-conjugated avidin (Amersham) were performed in TBST-3%BSA. After extensive washing with TBST, blots were developed with an enhanced chemiluminescence (ECL) -kit (Amersham). The blots were covered for one minute with a mixture of solution 1 and 2 (1:1; 1 ml/20 cm<sup>2</sup>). Excess solution was eliminated and the blot exposed to a film (Amersham). Films were developed in an automatic developer (Kodak).

## **2.11 Protein determination**

For the determination of protein concentrations, the "DC Protein Assay" (Biorad) was used, which is based on the Lowry assay. Mainly due to tyrosine and tryptophane rests, proteins reduce folin in an alkaline copper tatrare solution. The reduction product of folin has a characteristic blue colour with a maximum absorbency at 750nm which can be quantified

with a photospectrometer (Shimazu). To 10 $\mu$ l lysate 75 $\mu$ l of a 1:50 mixture of solution A and S was added. After vortexing, 600 $\mu$ l solution B was added, samples were mixed and the absorbency read after 15 minutes.

## **2.12 Preparation of sub-cellular fractions**

Disrupting cells by means of cell shearing or homogenisation often leads to nuclear protein loss. In order to assess nuclear translocation of MAP kinases and transcription factors in firmly adhered cells, which is believed to be a key step linking signal transduction initiated at the plasma membrane to gene transcription (see Introduction), sub-cellular fractions were therefore prepared as follows: cytosolic proteins of adhered or suspended cells were selectively extracted with low concentrations of Digitonin in extraction buffer (0.005% in HEPES 25mM pH 7.6, 90mM KCH<sub>3</sub>COO, 2.5mM MgCl<sub>2</sub>, 1mM EGTA, 12mM Glucose plus Phosphatase and Protease Inhibitors; see Lysis buffer) for 10 minutes on ice and the supernatants were collected. Remaining cytosolic proteins were washed away with extraction buffer. Nuclear proteins were extracted by osmotic disruption of nuclei (0.3M NaCl in extraction buffer) for at least 10 minutes on ice and the supernatant was collected. Finally, after additional washings, membrane proteins were extracted with extraction buffer containing 1% NP40. Fractions were >95% pure as measured by immunoblotting with anti-Lactate dehydrogenase (LDH), anti-pRb and anti-p56<sup>lck</sup> antibodies as markers for cytosolic, nuclear and membrane-associated proteins, respectively (see Figure 3.4b). LDH is a routinely used cytosolic marker. pRb contains a nuclear localization sequence and interacts in G<sub>0</sub>/G<sub>1</sub> with DNA-bound transcription factors. It is therefore a sensitive and reliable marker of nuclear protein loss in G<sub>0</sub> and G<sub>1</sub>. P56<sup>lck</sup> was preferred to a transmembrane receptor as a marker of membrane-associated proteins since it is only anchored to the plasma membrane with its myristylated aminoterminal and is therefore a more sensitive marker of membrane protein contaminations.

## **2.13 Immunoprecipitation**

Cells stimulated in various ways were lysed in Ip-buffer (Tris-Cl 10mM pH 7.4, 1% NP-40, 0.5% Desoxycholate, 0.1% SDS, 0.15M NaCl, 1mM EDTA, 10mM NaF, 10mM P207, 0.5mM Na<sub>3</sub>VO<sub>4</sub>, 100 $\mu$ g/ml PMSF, 1 $\mu$ g/ml Aprotinin and Leupeptin), protein concentration determined (see 2.11) and equal amounts of protein pre-cleared with Pansorbin cells which had been saturated with normal rabbit serum. The protein was precipitated with 1 $\mu$ g polyclonal rabbit antibodies pre-bound to Protein A-Sepharose for at least one hour at 4°C (Sigma) and the pellet washed three times in Ip-buffer.

## 2.14 In vitro kinase assays

JNK Kinase assay was performed as described previously (Hibi et al., 1993): Cells were lysed in JNK-Lysisbuffer (20mM HEPES pH 7.5, 0.3M NaCl, 1.5mM MgCl<sub>2</sub>, 0.2mM EDTA, 0.1% NP-40, 0.5mM DTT, 20mM β-glycerophosphate, 0.1mM Na<sub>3</sub>VO<sub>4</sub>, 100μg/ml PMSF, 1μg/ml aprotinin and leupeptin) and incubated for 10 minutes on ice. 3 volumes JNK-Dilutionbuffer (20mM HEPES pH 7.5, 2.5mM MgCl<sub>2</sub>, 0.1mM EDTA, 0.05% NP-40, 0.5mM DTT, 20mM β-glycerophosphate, 0.1mM Na<sub>3</sub>VO<sub>4</sub>, 100μg/ml PMSF, 1μg/ml aprotinin and leupeptin) were added and lysates cleared by centrifugation. 2-5μg of bacterially expressed, purified GST-c-Jun(1-79) (see 2.8) were incubated with the obtained whole cell extracts containing equal amounts of protein for 4 hours in the cold room. The pellet was washed in JNK-Washbuffer (20mM HEPES pH7.5, 50mM NaCl, 2.5mM MgCl<sub>2</sub>, 0.1mM EDTA, 0.05% NP-40) and resuspended in JNK-kinase buffer (20mM HEPES pH 7.7, 20mM MgCl<sub>2</sub> 2mM DTT, 20mM β-glycerophosphate, 20mM p-nitrophenylephosphate, 0.1mM Na<sub>3</sub>VO<sub>4</sub>, 20μM ATP) containing 5μCi (γP32)-ATP (Du Pont). The kinase reaction was carried out for 20 minutes at 37°C and stopped with sample buffer. In order to measure the activity of the p38 MAP kinase, the kinase was immunoprecipitated (see 2.13), the pellet was equilibrated in JNK-kinase buffer and then resuspended in JNK kinase buffer containing 5μCi (γP32)-ATP and 10μg myelin basic protein (Sigma). The kinase reaction and analysis by SDS-PAGE was performed as described for JNK. The activity of the src-kinases p56<sup>lck</sup> and p59<sup>fyn</sup> were assessed as follows: Both kinases were immunoprecipitated as described in 2.13 and the beads washed with src-kinases Washbuffer (10mM Tris-Cl pH 7.4, 0.15M NaCl, 0.1% NP-40) and resuspended in src-kinase buffer (10mM Tris-Cl pH 7.4, 10mM MgCl<sub>2</sub>, 10μM ATP, 5μCi (γ32)-ATP) containing 10μg enolase as an exogenous substrate. Samples were incubated for 5 minutes at room temperature, sample buffer was added and a SDS-PAGE performed as described (see 2.11). The activities of CDK2, 4 and 6 were assessed as described (34). Briefly, 10<sup>7</sup> cells stimulated in various ways were washed, resuspended in CDK-Lysisbuffer (50mM HEPES pH 7.5, 150mM NaCl, 1mM EDTA, 2.5mM EGTA, 1mM dithiothreitol, 0.1% Tween20, 0.1mM Na<sub>3</sub>VO<sub>4</sub>, 1mM NaF, 10mM β-glycerophosphate, 1mM sodium pyrophosphate plus protease inhibitors), sonicated for 20 seconds and the nuclei were spun down. The kinases were immunoprecipitated for 2 hours at 4°C with 1μg of polyclonal rabbit anti-CDK2, -CDK4 and -CDK6 antibodies (Santacruz) from the obtained pre-cleared lysates containing equal amount of proteins. The samples were washed twice in CDK-Lysisbuffer and once in CDK-Kinasebuffer (50mM Hepes pH 7.5, 10mM MgCl<sub>2</sub>, 5mM MnCl<sub>2</sub>, 1mM dithiothreitol). The kinase reaction was carried out in 30μl CDK-kinase buffer containing 1μg of recombinant GST-p53 (CDK2) or GST-pRb (CDK4

and 6) protein (Santacruz) and 10 $\mu$ Ci ( $\gamma$ P32)-ATP for 30 minutes at 37°C and was stopped by the addition of sample buffer. The samples were boiled and a standard SDS-PAGE performed. The gel were fixed for 2 hours with at least three changes of fixing solution (35% ethanol and 10% acetic acid) to reduce the background caused by not incorporated radioactivity, dried and exposed to an X-ray film (Amersham, Buckinghamshire, England).

## **2.15 RNA preparation and northern blotting**

Northern Blotting was performed to quantify the transcription of the immediate early genes *c-jun* and *c-fos*.

### **a) RNA preparation**

Total RNA was isolated from T cells with the "Ultraspec RNA system" (Bioctex), which contains a denaturing 14 molar solution of guanidine salts and urea. 5x 10<sup>7</sup> cells per sample were lysed with 1ml of ultraspec reagent. After 5 minutes of incubation on ice 200 $\mu$ l Chloroform were added, samples were vortexed and again incubated for 5 minutes on ice. Samples were then centrifuged for 15 minutes at 12000g at 4°C. The upper, aqueous phase containing the RNA was carefully taken, an equal volume of isopropanol added and samples were incubated for 10 minutes at 4°C. After centrifugation at 12000g at 4°C for 10 minutes, a white pellet of RNA precipitated and was washed twice with 75% ethanol by vortexing and centrifuging for 5 minutes at 4°C and 7500g. The pellet was air-dried and suspended in 50 $\mu$ l RNase-free water. The concentration of the obtained RNA was estimated by OD measurement with a spectrophotometer at 260nm and revealed that about 20 $\mu$ g RNA were obtained per sample. The OD measurement was controlled with a gel and ethidium bromide staining.

### **b) Gel separation and transfer of RNA**

Total RNA (15 $\mu$ g) was denatured in 10mM 3-(N-Morpholino)propane-sulfonic acid (MOPS), 4mM sodium acetate, 0.5mM EDTA pH8, 6.5% formaldehyde, 5% formamide for 15 minutes at 65°C, one tenth volume of RNA sample buffer was added (50% glycerol, 1mM EDTA, 0.25% bromphenole blue, 0.25% xylene cyanol) and RNA was separated on a 1.2% agarose gel containing 15% formaldehyde and RNA running buffer (20mM MOPS, 8mM sodium acetate, 1mM EDTA pH 8) in the same buffer. The RNA was then transferred on a nylon membrane (Hybond N+, Amersham) by capillarity and fixed with ultraviolet radiation.

### **c) Preparation of radioactive labelled DNA-probes**

The plasmids *c-jun*-pTZ18R and *c-fos*-Deg14 (Bravo Laboratory), containing the complete coding region of the proto-oncogenes *c-jun* and *c-fos* were cut with EcoRI or EcoRI and SphI, respectively, and the fragments

were gel-purified (see 2.6). The obtained fragments were radioactively labelled with the “rediprime DNA labelling system” (Amersham). The purified, heat-denatured fragment was added to the lyophilized compounds (dTTP, dATP, dGTP, random primers, Kleenow-polymerase, ATP, Mg<sup>2+</sup>) and resuspended, 10 $\mu$ Ci { $\alpha$ <sup>32</sup>P}-dCTP were added and samples were incubated for 10 minutes at 37°C. The reaction was stopped by the addition of 5 $\mu$ l of 0.2M EDTA, the probe isolated from free nucleotides by gel filtration (Sephadex G-50 columns, Pharmacia) and the purified probe was denatured at 95°C for 5 minutes.

#### **d) Hybridisation and autoradiography**

In order to block non-specific binding of DNA, the membrane was pre-hybridized for at least one hour at 65°C with 100 $\mu$ g/ml salmon sperm DNA in hybridisation buffer (125mM Na<sub>2</sub>HPO<sub>4</sub>, 1mM EDTA, 0.3% H<sub>3</sub>PO<sub>4</sub>, 0.25M NaCl, 7% SDS, 1% BSA, 1% polyethylenglycole) under agitation. The labelled probe was added and the membranes were incubated at least for 12 hours. The membranes were washed for 30 minutes in 2x SSC (2x 0.15M NaCl, 15mM sodium citrate), 0.1% SDS, 0.5x SSC, 0.1% SDS and 0.1x SSC, 0.1% SDS at 65°C. The membrane was exposed to a X-ray film (Kodak) at -80°C with an intensifying screen and films were developed with a developer machine.

## **2.16 Enzyme-Linked Immuno-Sorbent Assay (ELISA)**

ELISAs were used to measure either Interleukin-2 (IL-2) from the culture supernatant from stimulated T cells (**a**) or to test the reactivity of the engineered GST-ICAM fusion protein with a monoclonal anti-CD54 antibody, which recognizes the binding site of ICAM-1 (**b**).

#### **a) Interleukin-2**

IL-2 released by activated T cells was measured using a protocol previously described by Dr H. Gallatti (INTEX, Switzerland). Microtiter plates were coated with the monoclonal anti-human IL-2 antibodies 3D5 and 7B1 (Roche) at 5 $\mu$ g/ml in 100mM sodium phosphate pH 4.5 for 24 hours at room temperature. The plates were washed three times with deionized water and saturated with 1% BSA in 200mM Tris pH 7.5 for 24 hours at 4°C. The supernatant of T cells stimulated at 5x 10<sup>6</sup> cells/ml for various time periods or a reference concentration (1ng/ml) of recombinant IL-2 (Proleukin, Chiron Corporation) were serially diluted and incubated in the pre-coated microtiter plates in 1% FCS and the presence of the soluble, peroxidase-coupled monoclonal antibody 13A6 (Roche) at 0.06mg/ml final concentration. After five washes with deionized water containing 0.05% Tween20 the amount of bound peroxidase was enzymatically determined with tertamethyl-benzidine (1mM) - H<sub>2</sub>O<sub>2</sub> (2.5mM) as a substrate in 0.1M potassium citrate pH 4.1 containing 0.5% acetone and 4.5% ethanol for 10

minutes at room temperature. The reaction was stopped with 1M sulphuric acid and the colour intensity was read at 450nm with a spectrophotometer (Shimadzu). The concentration of IL-2 in the supernatants can be calculated from the standard curve multiplying with the corresponding dilution factor. The ELISA was routinely performed by Dr. Paola Panina (Roche).

#### **b) GST-ICAM**

The reactivity of the GST-ICAM fusion protein with the adhesion-blocking anti-ICAM-1 antibody (clone 15.2, Ancell) was tested with an ELISA to confirm that the expressed fusion protein contained the LFA-1 binding site. GST-ICAM, GST-Jun or GST alone were serially diluted in PBS (10 $\mu$ g/ml-78ng/ml). ELISA plates (Falcon) were incubated with 100 $\mu$ l/well for 16 hours at 4°C in a humid atmosphere. Plates were washed three times with PBST (PBS, 0.2% Tween20) and were then saturated with 1% BSA in PBS for 1 hour at 37°C to prevent non specific binding of the primary antibody. The primary anti-ICAM antibody was used at 5 $\mu$ g/ml in 0.1% BSA in PBS, 0.05% Tween20 and the incubation was performed at 37°C for 2 hours. After 5 washes with PBST, plates were incubated with an alkaline phosphatase conjugated goat anti-mouse Ig polyclonal antibody (Zymed) diluted 1:1000 in PBS, 0.1% BSA, 0.05% Tween20 for 30 minutes at 37°C. Plates were again washed 5 times. The bound conjugated antibody was revealed by incubating with 0.1M diethanolamine pH 8.8 containing 1mg/ml para-nitrophenyle-phosphate for 30 minutes at 37°C. The reaction was stopped with 1M NaOH. The phosphatase-cleaved substrate is yellow and can be quantified with a photometer at 405nm. The GST-ICAM but not the GST-Jun fusion protein or the GST alone were specifically bound by the anti-ICAM-1 antibody in a dose-dependent manner (not shown).

### **2.17 Electroporation of primary T cells**

Since no specific inhibitor of the JNK pathway has been developed yet, it was tried to competitively inhibit JNK with the GSTc-Jun(1-79) fusion protein by electroporating cells in the presence of the inhibitor as described previously (Su et al., 1994; Szamel et al., 1993). T cells (10<sup>6</sup>) were resuspended in intracellular buffer (10mM HEPES pH 7.6, 5mM MgCl<sub>2</sub>, 120mM KCl, 10mM EGTA, 8.2mM CaCl<sub>2</sub>) containing 10 $\mu$ g/ml GST-Jun or GST as a control. Cells were then electroporated at 250 $\mu$ F and 300V and left on ice for 5 minutes; they were then diluted in RPMI supplemented with 10% FCS and were allowed to recover for 1 hour at 37°C. Cells were >90% viable, they were subsequently stimulated and proliferation was assessed after 72 hours of stimulation with propidium iodide staining.

## **2.18 Electrophoretic mobility shift assay (EMSA)**

EMSAs allow the detection of binding activity of nuclear extracts specific for radioactive labelled Oligonucleotides mimicking promoter fragments due to the retarded migration of the oligonucleotides in a non-denaturing electrophoresis. Preincubating nuclear extracts with antibodies specific for selected transcription factors allow further the identification of the proteins binding to the Oligonucleotides. It is assumed that the assembly of the highly co-operative multi-protein complex at the IL-2 promoter probably requires the simultaneous occupancy of all sites; the lack of even one single factor may abolish the formation of the whole enhancer complex (Jain et al., 1995). In order to allow the co-operative binding of transcription factors it was therefore avoided to separate neighboured consensus sites.

### **a) nuclear extracts**

Cells stimulated in various ways were washed with PBS and cytosolic proteins were extracted as described in 2.12. Nuclear proteins were subsequently extracted with Totex buffer (20mM HEPES pH 7.8, 350mM NaCl, 10mM NaF, 20% glycerole, 1% NP-40, 1mM MgCl<sub>2</sub>, 0.35mM EDTA, 0.05 mM EGTA, 1mM DTT plus phosphatase and protease inhibitors). Equal amounts of proteins were loaded on a SDS-PAGE and the purity of the cytosolic and nuclear fractions were controlled by immunoblotting as described in 2.12.

### **b) Construction and radioactive labelling of the oligonucleotide probes**

The four most important fragments of the human IL-2 promoter for the inducible IL-2 transcription in primary T cells (Hughes and Pober, 1996) were used for the shift assays: The NF- $\kappa$ B consensus site 5'-CAGAAAGATTTTCACCTA-3', the CD28-RE/AP-1-site 5'-CGGTTTAAAGAAATTCCAAAGAGTCATCAGA-3', the proximal NF-AT site 5'-GTGATCAGAAGAGGAAAAATGAAGGT-3' and the AP-1/OCT-site 5'-TTGAAAATATGTGTAATATGTAAAACATTTTGA-3'. The oligonucleotides were synthesised with overhangs of three nucleotides containing one guanosine rest, thus allowing the radioactive labelling of the annealed oligonucleotides with the Kleenow polymerase in the presence of radioactive dCTP. The complementary oligonucleotides were together boiled for 5 minutes and were allowed to cool down slowly. Then 16pmol of annealed oligonucleotide were incubated with 40 $\mu$ Ci of  $\{\alpha\text{-p}^{32}\}$  dCTP, 0.1mM dTTP, dGTP and dATP, 1 unit Kleenow enzyme in 1x Kleenow buffer (Promega) in 50 $\mu$ l for 30 minutes at room temperature. Not incorporated nucleotides were separated with a G-50 column (Nick column, Pharmacia). The activity of the obtained probes were controlled with a liquid scintillation analyzer (Packard).

**c) Formation and electrophoretic separation of the DNA/protein complexes**

1-10 $\mu$ g nuclear extract in 2 $\mu$ l were pre-incubated with 2 $\mu$ l Polydeoxyinosine-deoxycytidine (1mg/ml, Pharmacia), 1 $\mu$ l BSA (10mg/ml, molecular biology grade, MBI Fermentas), 1 $\mu$ l Desoxycholate (4%) and 4 $\mu$ l of 5x binding buffer (100 mM Tris-HCl pH 7.5, 5mM EDTA, 0.5% NP-40, 20% glycerole) in a final volume of 19 $\mu$ l for 15 minutes at 4°C. For supershift analysis, 1 $\mu$ l of a purified anti-RelA (C-20), -p50(C-19) c-Rel (N), -NF-AT (K-18), -c-Jun (KM1) or -c-Fos (K-25, 2mg/ml, Santacruz) antibodies were pre-incubated with the nuclear extract for 30 minutes at 4°C. Then 1 $\mu$ l of a labelled oligonucleotide (100.000-200.000 CPM) was added and the samples were incubated for 15 minutes at room temperature. The samples were loaded on a non-denaturing 0.5x TBE (8.9mM Tris, 8.9mM boric acid, 0.2mM EDTA) 5% acrylamide-gel, which had been pre-run at 200 Volt for 2 hour at 4°C. The gel separation was carried out at 4°C for 90 minutes at 220 Volts. The gel was dried and exposed to a film.