2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Acetic acid (CH_3CO_2H)	Merck, Darmstadt
Acrylamid	Sigma, Deisenhofen
Agar	Difco, Detroit, USA
Agarose	PEQLAB, Erlangen
Ammonium acetat (CH_3COONH_4)	Riedel-de Haen, Seelze
Ammonium persulfate ($H_8N_2O_8S_2$)	Sigma, Deisenhofen
Ammmonium sulfate	ICN Biomedicals, Cleveland
Anhydrotetracyclin	Sigma, Deisenhofen
L-Arginin	Sigma, Deisenhofen
Bacto-peptone	Roth, Karlsruhe
Bacto-tryptone	Roth, Karlsruhe
Bacto-yeast extract	Roth, Karlsruhe
Benzonase	Sigma, Deisenhofen
Bromophenol blue	Sigma, Deisenhofen
BSA	Biomol, Hamburg
Coomassie Brilliant Blue R-250	Sigma, Deisenhofen
Deoxycholate	Sigma, Deisenhofen
Dithiothreitol	Sigma, Deisenhofen
Dnase I	Roche, Mannheim
dNTPs (100 mM)	Invitrogen, Karlsruhe
Dpn I endonuclease	Stratagene, California
DTT	USB, Cleveland
EDTA	Sigma, Deisenhofen
Ethanol	Merck, Darmstadt
Formalin	Merck, Darmstadt
Glucose	Sigma, Deisenhofen
Glutathione reduced/oxidized	Sigma, Deisenhofen
Glutaraldehyde	Sigma, Deisenhofen
Glycerol	Roth, Karlsruhe
Glycine	Biomol, Hamburg
Guanidinium HCl	Serva, Heidelberg
Hydrochloric acid (HCl)	Merck, Darmstadt
IPTG	AppliChem, Darmstadt
Isopropanol	Merck, Darmstadt
Lysozyme	Sigma, Deisenhofen

Methanol	Merck, Darmstadt
2-Mercaptoethanol	Sigma, Deisenhofen
Magnesium chloride ($MgCl_2$)	Merck, Darmstadt
Manganese (II) chloride ($MnCl_2$)	Merck, Darmstadt
Na_2EDTA	ICN Biomedicals, Cleveland
PBS	Invitrogen, Karlsruhe
Phosphoric acid (H_3PO_4)	Merck, Darmstadt
Phenol	Biomol, Hamburg
PMSF	Serva, Heidelberg
Potassium acetate (CH_3COOK)	Merck, Darmsatdt
Potassium chloride (KCl)	Merck, Darmstadt
Potassium dihydrogen phosphate (KH_2PO_4)	Merck, Darmtadt
Protein marker	Invitrogen, Karlsruhe
SDS	Serva, Heidelberg
Silver nitrate ($AgNO_3$)	Sigma, Deisenhofen
Sodium acetate (CH_3COONa)	Merck, Darmsatdt
Sodium azide (NaN_3)	Merck, Darmstadt
Sodium bicarbonate ($NaHCO_3$)	Merck, Darmstadt
Sodium chloride (NaCl)	Roth, Karlsruhe
Sodium citrate	Roth, Karlsruhe
Sodium dihydrogen phosphate (NaH_2PO_4)	Merck, Darmstadt
Sodium hydrogenphosphate (Na_2HPO_4)	Merck, Darmstadt
Sodium hydroxide (NaOH)	Merck, Darmstadt
Sodium lactate	Roth, Karlsruhe
Sodium pyruwate	Roth, Karlsruhe
Sodium thiosulfate	Sigma, Deisenhofen
Sucrose	Merck, Darmstadt
TEMED	Serva, Heidelberg
Trehalose dihydrate	Sigma, Deisenhofen
Triton X-100	Serva, Heidelberg
Tris	Roth, Karlsruhe
Tween-20	Sigma, Deisenhofen
Urea	Merck, Darmstadt
X-Gal	Biomol, Hamburg

2.1.2 Solutions, buffers and media

2.1.2.1 Inclusion body production, refolding and purification

Lysis Buffer (pH 8.0)	25 % Sucrose 1 mM EDTA 50 mM Tris
Detergent buffer (pH 8.0; without DTT)	200 mM NaCl 1 % Deoxycholate 1 % Nonidet-40 2 mM EDTA 20 mM Tris
Triton buffer (pH 8.0; without DTT)	100 mM NaCl 1 mM EDTA 0,5 % TritonX-100 50 mM Tris
Inclusion Body buffer (pH 7.5)	20 mM Tris 150 mM NaCl
Urea buffer (pH 7.5)	50 % (w/w) Urea 50 mM NaCl 20 mM Tris/HCl
Guanidinium HCl	6 M Guanidinium HCl 10 mM PBS pH 7.5
Refolding buffer (pH 7.5)	400 mM L-Arginin 2 mM EDTA 5 mM red. Glutathione 0,5 mM oxyd. Glutathione 100 mM Tris pH 8.0

FPLC buffer (pH 7.5)	20 mM Tris/HCl
	150 mM NaCl
	0,01 % Na-Azid

2.1.2.2 SDS-PAGE

13 % separating gel	375 mM Tris (pH 8.8)
	13 % acrylamide, 0.34 % bis-acrylamide
	0.1 % SDS
	0.1 % APS
	10 µl TEMED
5 % stacking gel	0.125 % Tris (pH 6.8)
	5 % acrylamide, 0.13 % bis-acrylamide
	0.1 % SDS
	0.1 % APS
	8 µl TEMED
Sample buffer - reducing conditions	7.5 % SDS (w/v)
	250 mM Tris (pH 8.0)
	0,25 % Bromophenol Blue (w/v)
	12.5 % 2-Mercaptoethanol (w/v)
	25 % Glycerol (w/v)
Sample buffer - nonreducing conditions	7.5 % SDS (w/v)
	250 mM Tris (pH 8.0)
	0,25 % Bromophenol Blue (w/v)
	25 % Glycerol (w/v)
10 x Running buffer	230 mM Tris (pH 8.3)
	2 M Glycine
	0.1 % SDS

2.1.2.3 Staining of protein gels

Coomassie staining buffer	10 % Acetic acid 40 % Ethanol 0,25 % Coomassie Brilliant Blue R-250
Decolour Solution	10 % Acetic acid 45 % Methanol
Drying Buffer	10 % Glycerol 20% Ethanol
Fixation Solution	50 % Methanol 12 % Acetic acid 0.018 % Formalin
Incubation Solution	0.8 mM Sodium thiosulfate
Ag incorporating Solution	10 mM Silver nitrate 0.028 % Formalin
Dyeing Solution	200 mM Sodium carbonate 0.018 % Formalin 2 % Incubation Solution
Fixation Solution II	50 % Methanol 12 % Acetic acid
Incubation Solution II	30 % Isopropanol 2 % Glycerol

2.1.2.4 Media for bacteria and agar plates

LB Medium (pH 7.5)	1.0% Bacto-tryptone (w/v) 0.5% Yeast extract (w/v) 1.0% NaCl
LB-Agar	1.0% Bacto-tryptone 0.5% Yeast extract 1.0% NaCl 1.5% Agar
SOC Medium	20 g Bacto-tryptone 5 g Bacto-yeast extract 0.5 g NaCl in 1 liter deionized water add 20 ml 1 M Glucose

IPTG/X-Gal plates: LB-Agar with 50 µg/ml ampicilin, 100 µM IPTG and 0.4 % X-Gal was poured into petri dishes. The dishes were stored at 4°C.

2.1.2.5 Frequently used buffers and solutions

Elution buffer	1.5 M NaCl 20 mM Tris/HCl (pH 7.5) 0.1 mM EDTA
PBS buffer	130 mM NaCl 7 mM Na_2HPO_4 4 mM NaH_2PO_4
PBT buffer	0.1% Tween-20 in PBS
TE buffer (p 8.0)	10 mM Tris/HCl (pH 8.0) 1 mM EDTA

2.1.3 Antibiotics

Stock solutions were prepared for the antibiotics. The stock solutions were then filtered through sterile disposable filters and stored at -20°C. When antibiotics were needed, in each case they were added after the autoclaved medium had cooled down to a temperature lower than 55°C.

	Master solution	Solvent	Final concentration
Ampicillin	50 mg/ml	H_2O	50 µg/ml

2.1.4 Bacterial strains

E. coli DH 10b	Invitrogen
E. coli TG1	Stratagene
E. coli JM 109	Stratagene
Origami (DE3)	Stratagene
BL21 (DE3)	Stratagene

2.1.5 Plasmids

p HN1	(Garboczi et al., 1992)
pASK-IBA6	IBA
pET23a(+)	Novagen

2.1.5 Synthetic oligonucleotide primers

The synthetic oligonucleotide primers used in this study were obtained from The Biopolymer Factory (Ulm, Germany) and dissolved in water to a final concentration of stock solution 100 pmol/µl.

B2705-04 D77S fw	5´-CCGAGAGTCCCTGCGGACC-3´
B2705-04 D77S rv	5´-GGTCCGCAGGGACTCTCGG-3´
B2705-04 V152E fw	5´-GGCCCGTGAGGCGGAGCAGC-3´
B2705-04 V152E rv	5´-GCTGCTCCGCCTCACGGGCC-3´

B2705-04 A211G fw	5´-GCTTCTACCCTGGGGAGATCACAC-3´
B2705-04 A211G rv	5´-GTGTGATCTCCCCAGGGTAGAAGC-3´
B2704-06 HD114/6DY fw	5´-CGGGTACGACCAGTACGCCTACG-3´
B2704-06 HD114/6DY rv	5´-CGTAGGCGTACTGGTCGTACCG-3´
pASK-Seq-For	5´-TAGAGTTATTTTACCACTCC-3´
pASK-Seq-Rev	5´-CGCAGTAGCGGTAAACGGC-3´

2.1.6 Synthetic peptides

The peptides were synthesized by ZEBiolabs Inc., Westfield, USA, and Alta Bioscience, University of Birmingham, according to standard procedures and purified by HPLC. All peptides were dissolved in 25 % DMSO to a concentration of 5 mg/ml and stored in aliquots at -20°C.

pLMP2	RRRWRRLTV (a viral-peptide, derived from Latent Membrane Protein 2 Antigen of Epstein-Barr virus; residues: 236-244)
pVIPR	RRKWRRWHL (a self-peptide, derived from vasoactive intestinal peptide type 1 receptor, residues: 400-408)
pEBV8TvE	RRIYDLITL (an immunodominant viral peptide: Epstain-Barr nuclear antigen 3C (EBNA3C) derived from Epstein-Barr virus, modified at position 8; 258-266; Sewart-Jones et al., 2005)
pNK	RRIKAIIHK (model peptide; Peruzzi et al., 1996)
npNK	RRIKAITLK (model peptide; Peruzzi et al., 1996)
np1NK	RRYQKSTEL (a self-peptide derived from human histone H3.3; 52-60; Jardetsky et al., 1991)
MV-F	RRYPDAVYL (a viral peptide derived from transmembrane fusion glycoprotein (F) of measles virus; 438-446; Binnendijk et al., 1992)

2.1.7 Kits

QuickChange Site-Directed Mutagenesis Kit	Stratagene
FastPlasmid Mini Kit	Eppendorf
Midi Plasmid Kit	JETstar
STABIL-PAC	Novexin

2.1.8 Laboratory materials

Aluminium foil	Amersham
Amicon Ultra	Millipore
Centricon-10, Amicon	Millipore
Petri dishes	Greiner
Pipette tips	Eppendorf
Microcentrifuge tubes	Eppendorf
Microcon columns	Millipore
Crystallographic 24-wells plates	Huntington Research

2.1.9 Sterilisation of solutions and equipments

All solutions that are not heat-sensitive were autoclaved at 121°, 10^5 Pa for 60 min (Webco, Bad Schwartau). Heat sensitive solutions were filtered through a disposable sterile filter (0.2 to 0.45 µm pore size). Plastic wares were autoclaved as above. Glass wares were sterilised overnight in an oven at 220°C.

2.1.10 Instruments

ÄKTA FPLC	Amersham Biosciences
Cell Disruptor B15	Branson
Centrifuge 5417R	Eppendorf
Electrophoresis Power Suply-EPS 3500	Pharmacia Biotech
Microscope C3040-ADL	Olympus
Multiporator	Eppendorf
Personal PCR Cycler	Biometra
Sorvall RC SC Plus	Sorvall
SpectraFlour Plus	Tecam
Spectrophotometr Ultraspec 2100 pro	Amersham Biosciences
SpeedVac concentrator Alpha 1-2	Christ
Thermomixer 5436	Eppendorf

2.2 Methods

2.2.1 Designing primers for *in vitro* mutagenesis

Primers for site directed mutagenesis were designed on the basis of the HLA-B*2705 sequence (NCBI accession number GI: 5705951; http://ncbi.nlm.nih.gov). For production of B*2704, six primers were used (three forward and three reverse), each with single mutation, which resulted in three amino acids exchanges. B*2706 subtype was made on the basis of the B*2704 subtype using one forward and one reverse primer, which mutated two polymorphic residues differentiating B*2704 from B*2706.

2.2.2 Site-directed mutagenesis

In vitro site-directed mutagenesis is a technique used for carrying out vector modification and for studying protein structure-function relationships and gene expression. This method allows making point mutations, switching amino acids, and deleting or inserting single/multiple amino acids using supercoiled double-stranded DNA (dsDNA), vector with a gene of interest and two synthetic oligonucleotide primers containing the desired mutation. The *PfuTurbo* DNA polymerase extends the primers, each complementary to opposite strands of the vector. This process generates a mutated plasmid containing staggered nicks. The paternal DNA template is methylated and therefore can be easily digested by the *Dpn I* endonuclease (target sequence: 5´-Gm^{6}ATC-3´). An *in vitro* site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis Kit from Stratagene. The first step in this method is a mutant strand synthesis reaction (thermal cycling).

The **control reaction** was prepared as indicated below:

10 x reaction buffer	5 µl
pWhitescript 4.5 kb control plasmid	2 µl (10 ng)
oligonucleotide control primer #1	1,25 µl (125 ng)
oligonucleotide control primer #2	1,25 µl (125 ng)
dNTP mix	1 µl
double destilled water to final volume 50 µl	
PfuTurbo DNA polymerase (2,5 U/µl)	1 µl

The **sample reaction** was prepared as indicated below:

10 x reaction buffer	5 µl
dsDNA template	5-50 ng
oligonucleotide primer #1	125 ng
oligonucleotide primer #2	125 ng
dNTP mix	1 µl
double destilled water to final volume 50 µl	
PfuTurbo DNA polymerase (2,5 U/µl)	1 µl

Cycling parameters:

Segment	**Cycles**	**Temperature**	**Time**
1	1	95°C	30 sec
2	12	95°C	30 sec
		55°C	1 min
		68°C	1 min/kb of plasmid length

After the mutant strand synthesis reaction, *Dpn I* digestion of the amplification products was employed. To each amplification reaction, 1 µl of the *Dpn I* restriction enzyme (10U/µl) was added. The reaction mixture was mixed gently by pipetting and spinned down in a microcentrifuge for 1 minute. Digestion of the parental supercoiled dsDNA was performed at 37°C for 1 hour. 10 µl of the *Dpn I*-treated DNA has been used for transformation of the competent cells.

2.2.3 Isolation of nucleic acids

2.2.3.1 Small-scale isolation of plasmid DNA

Plasmid DNA was isolated according to Sambrook et al. (1989).

A mini preparation of plasmid DNA was performed using the FastPlasmid Mini kit from Eppendorf. A single *E. coli* colony was inoculated in 5 ml of LB medium with the appropriate antibiotic and incubated in a shaker for 16 hrs at 37°C with a speed of 160 rpm. 1 ml of the saturated culture was used for making a glycerol stock, and 1.5 ml was centrifuged at 12000 x *g* for 1 min. The pellet was resuspended in 400 µl of ice-cold Complete Lysis Solution and vortexed for a full 30 seconds. The lysate was incubated at RT for 3 min and after this time

transported to a spin column assembly. The column was centrifuged for 30-60 seconds at maximum speed. The pellet was washed with 400 µl of Diluted Wash Buffer. After drying the column by one additional centrifugation, it was placed in a collection tube and the DNA was eluted by adding 50 µl of Elution Buffer directly to the center of the spin column membrane.

2.2.3.2 Large-scale preparation of plasmid DNA

A single colony was inoculated in 2-5 ml LB medium with the appropriate antibiotic as a pre-culture for 8 hrs in a shaker at 37°C. The pre-culture was diluted 1:100 fold in 100 ml LB medium with appropriate antibiotic and incubated overnight at 37°C with shaking. The saturated culture was centrifuged at 6000 x *g* for 15 min at 4°C. The pellet was resuspended in 4 ml of solution E1 and cells were lysed with E2, incubated at RT for 5 min and neutralized with E3 solution. The solution was centrifuged at 15000 x *g* for 10 min at 20°C. Meanwhile, the column that was provided with the preparation kit was equilibrated with 10 ml of E4 solution. After centrifugation, the lysate was layered onto this equilibrated column allowing the DNA to bind with the resin present at the bottom of the column. The column was then washed twice with 10 ml of E5 solution. Finally, the DNA was eluted with 5 ml of E6 solution. To precipitate the DNA, 3.5 ml of isopropanol was added and mixed thoroughly and centrifuged at 15000 x *g* for 30 min at 4°C. The DNA pellet was washed with 70 % ethanol and dissolved in 100 µl of TE.

Solution E1:	50 mM	Tris
	10 mM	EDTA pH 8.0
Solution E2:	200 mM	NaOH
	1 %	SDS (w/v)
Solution E3:	3.1 M	Potassium acetate, pH 5.5
Solution E4:	600 mM	NaCl
	100 mM	Sodium acetate
	0.15 %	TritonX-100
Solution E5:	800 mM	NaCl
	100 mM	Sodium acetate, pH 5.0

Solution E6: 1250 mM NaCl
100 mM Tris, pH 8.5

2.2.4 Determination of nucleic acid concentrations

The concentration of nucleic acids was determined spectrophotometrically by measuring the absorption of the samples at 260 nm and 280 nm. The quality of nucleic acids i.e. contamination with salts and proteins was checked by measurements at 230, 280 and 320 nm.

2.2.5 Gel electrophoresis

Gel electrophoresis is the technique by which mixtures of charged macromolecules, especially nucleic acids and proteins are separated in an electrical field according to their mobility which is proportional to the ratio of macromolecule's charge and mass.

2.2.5.1 SDS-PAGE

SDS-PAGE is a method for separating proteins within a sample. The proteins are denaturated and rendered monomeric by boiling in the presence of reducing agents (β-mercaptoethanol or dithiothreitol) and a negatively charged detergent (SDS). The proteins, which normally differ according to their charges, are all coated with the SDS molecules. Hence, all the proteins in the sample become negatively charged and achieve constant charge to mass ratio. In this way, the separation is according to the size of the proteins. SDS-PAGE employed here consisted of two gels: a separating gel and a stacking gel. In conducted experiments, a 13 % separating gel and a 5 % stacking gel were used. The samples were boiled in sample buffer for 10 min at 95°C before loading onto the gel (Laemmli, 1970). The gel was exposed for 1 hr to 15 mA, then to a constant current of 30 mA (approximately 160 V) as long as required for the separation of the protein of interest.

2.2.6 Transformation of competent bacteria

2.2.6.1 Transformation of $CaCl_2$ competent *E. coli* cells

(Ausubel et al., 1994)

Transformation of the bacteria was done by gently mixing one aliquot of competent bacteria with 10 µl of ligation mixture. After incubation for 30 min on ice, bacteria were heat shocked for 20 s at 37°C and cooled down for 2 min on ice. After adding 600 µl of SOC or LB medium (see 2.1.2.6) bacteria were incubated at 37°C in a shaker at 160 rpm for 1 hr and plated out on ampicillin LB-agar plates or whenever required on IPTG/X-Gal plates.

2.2.6.2 Transformation of electrocompetent *E. coli* cells

Transformation was done by adding 5 µl of ligation mixture to 25 µl of competent cells thawn on ice. The content of the tube was transported to the cuvette and electroporated. The cuvette was immediately transferred to ice and 500 µl of LB medium with ampicillin was added. The tubes with transformation were shaked at 37°C for 1hr following spreading of 10-50 µl from the transformation on LB-agar plates containing X-Gal or IPTG.

2.2.7 Methods for analysis of proteins

2.2.7.1 Protein expression in the form of inclusion bodies

Inclusion bodies are insoluble forms of proteins deposited in *E. coli* cells. Transferring inclusion body proteins to their native state involves two steps: (a) solubilisation and (b) refolding of the protein. The yield of natively folded protein is often substantially reduced due to aggregation or misfolding; it may, however, be improved by certain additives to the folding buffer.

300 ml of LB + Amp was inoculated with 1-2 ml of o/n pre-culture (*E. coli*) and shaked (200 rpm) for 2 hrs at 37°C. After the culture reached OD_{560} ~ 0.3-0.5 it was induced to produce the appropriate protein (IPTG, 1:1000 of 400 mM stock for pHN vector; anhydrotetracyclin, 1:10000 of 2 mg/ml stock for pASK6-IBA vector). The expression of the protein was conducted either at 37°C for 4 hrs or at RT o/n when needed for improving of the product yield. The culture was centrifuged 20 min at 2700 x *g* at 4°C. The supernatant was discarded and the pellet was

resuspended in 10 ml of lysis buffer. After adding 100 ml of PMSF and 500 ml lysozyme the lysate was incubated 30 min on ice. 10 µl of DNase I, 100 µl of 1M $MgCl_2$ and 10 µl of 1M $MnCl_2$ were added and the mixture was sonicated with Microtip (2 x 15 pulses with duty cycle: 30%, output control: 3.5). For removing DNA from KIR-HLA complexes, bensonase was used (30 min incubation at 37°C). The lysate was centrifuged for 10 min at 4°C, 10000 x *g*; for preparation of further inclusion bodies, the pellet was resuspended in 10 ml of Detergent buffer and 20 µl of 1M DTT, sonicated and centrifuged as described above. In the next step the pellet was washed with 10 ml Tritonbuffer (optionally with 20 µl of 1M DTT), sonicated, incubated 10 min on ice and centrifuged.

After additional three washings with 10 ml of Tritonbuffer (Sonication + centrifugation like above, without incubation on ice), the pellet was resuspended in 4 ml of InB-Buffer, and 40 µl of 1M DTT was added. Aliquots (1 ml) were stored in -20°C.

2.2.7.2 Refolding of proteins

A desired amount of inclusion bodies was refrozen and centrifuged 10 min at 4°C, with 10000 x *g*. The supernatant was discarded and the pellet was dissolved in 1 ml 8 M urea buffer. Shaking for 1 hr at RT was employed following 20 min centrifugation at RT at 10000 x *g*. The supernatant was transported to a Falcon tube, and the concentration of the protein was determined (OD_{280}) at dilution 1:100. Values in mg/ml corresponding to 1OD for β_2m heavy chain and KIR3DL1 were calculated from the extinction coefficient. The extinction coefficient factor was calculated for each protein separately using the ProtParam program of ExPasy (available online: http://www.expasy.org/tools/protparam.html). The concentration of the protein was recalculated on the basis of these values.

1OD – 0,61 mg/ml for β_2m
1OD – 0,46 mg/ml for HC
1OD – 0,59 mg/ml for KIR3DL1

2.2.7.3 Reconstitution of proteins

For 200 ml of reconstitution buffer the following amounts of inclusion bodies were added:
- 6 mg of HC
- 5 mg of β_2m
- 2 mg of peptide

for reconstitution of KIR3DL1 with HLA-B27:
- 8 mg of KIR3DL1 in addition

In standard reconstitution set-ups the peptide was added first, then the pre-mix containing HC and β_2m, and KIR3DL1. Set-ups were kept at 4°C for approximately 2 weeks. Deviations from this protocol are described for each reconstitution in the Results section. After two weeks the refolding mixture was concentrated to 500 µl using Amicon tubes. The sample was centrifuged for 10 minutes at 10000 x *g* and applied to the Fast Performance Liquid Chromatography (FPLC) machine for purification by size exclusion chromatography (Materials and Methods 2.2.7.4).

2.2.7.4 Protein purification by Fast Performance Liquid Chromatography (FPLC)

Fast Performance Liquid Chromatography, referred to as FPLC, is a form of column chromatography used to separate or purify proteins from complex mixtures. It is very commonly used in biochemistry and enzymology. Columns used with an FPLC can separate macromolecules based on size, charge distribution, hydrophobicity, or biorecognition (as with affinity chromatography). In this study size exclusion chromatography (SEC) was used. It is a chromatographic method in which particles are separated based on their size, or in more technical terms, their hydrodynamic volume. It is usually applied to large molecules or macromolecular complexes such as proteins. The underlying principle of SEC is that particles of different sizes elute (filter) through a stationary phase at different rates. Provided that all particles are loaded simultaneously or near simultaneously, particles of the same size should elute together. This is achieved with a column, which consists of a hollow tube tightly packed with extremely small porous polymer beads designed to have pores of different sizes.

For purification of HLA:peptide complexes we used a Superdex 75 filled column, for HLA:β_2m:peptide-KIR complexes a Superdex 200 filled column was used. As the solution travels down the column, some particles enter into the pores. Larger particles cannot enter into as many pores. The larger the particles are the less overall volume to traverse over the length of the

column, and the faster the elution is. In simple manually operated columns the eluent is collected in constant volumes, known as fractions. The more similar the particles are in size, the more likely they will be in the same fraction and not detected separately. The collected fractions are examined by spectroscopic techniques to determine the concentration of the particles eluted.

Both columns were calibrated with different proteins of known size in order to establish the elution profile of each column. This enables collecting those fractions which contain the desired protein complex.

2.2.7.5 Protein preparation for sequence analysis by MALDI-TOF/TOF

The proteins which were analyzed by Mass Spectrometry were run on the 13 % separating SDS gel as described in section 2.2.5.1. The gels were prepared very carefully to eliminate any contaminations which could have an influence on the results of MALDI analysis. After electrophoresis, the gel was stained using Coomassie. The bands which corresponded to the protein and should be applied for Mass Spectrometry were then carefully cut out of the gel and delivered in sterile Eppendorf tubes to the Mass Spectrometry Group (Dr. E. Krause) in the Leibniz-Institut für Molekulare Pharmakologie, Berlin-Buch, where MALDI-TOF/TOF spectra were taken.

2.2.8 Crystallographic methods

To determine the three dimensional structure the desired molecule has to occur in the crystalline state. A single molecule in solution has insufficient scattering power whereas in a crystal a particular arrangement of atoms is repeated. The oldest and most precise method of X-ray crystallography is single-crystal X-ray diffraction. This technique has three basic steps. The first one is to obtain an adequate crystal of investigated protein or other organic/inorganic biological molecule. Since they do not occur naturally as crystals, therefore they are placed in solution and allowed to crystallize over days, weeks, or months through vapor diffusion. The crystal should be pure with regular structure and appropriate size. During the second step the crystal is placed in an intense beam of monochromatic X-rays. This process produces a pattern of reflections and sets of data are collected. Finally these data are processed to produce and refine a model of the atoms arrangement within the crystal.

2.2.8.1 Protein crystallization

All crystallization trials were performed by vapor diffusion at 18°C using the hanging drop technique (Ducruix and Giegé, 1992). The hanging drop vapor diffusion technique is the most popular method for the crystallization of macromolecules. The principle of this method is as follows. The pure protein under study is in a buffer that preserves the native form of the protein. To this solution a precipitating agent is added that supersaturates the protein until it crystallizes or precipitates from this solution. A drop composed of a 1:1 mixture of the protein sample and a solution containing buffer and precipitant is placed in vapor equilibration with a larger reservoir containing the same buffers and precipitants. To achieve equilibrium, water vapor leaves the protein drop and eventually ends up in the reservoir. As water leaves the drop the relative supersaturation of the protein solution increases. Equilibration is reached when the reagent concentration in the drop is approximately the same as that in the reservoir. Since the system is in equilibrium, these optimum conditions are maintained until the crystallization (or precipitation) of the protein has been completed.

The gelfiltration purified protein complex, HLA:β_2m:peptide or HLA:β_2m:peptide with KIR3DL1 in FPLC buffer was concentrated to 7 -12 mg/ml using Centricon-10 concentration tubes and was used for preparing crystallization set-up. Crystallization conditions for HLA complexes were identified using the PEG 6000-pH and PEG 8000-pH screens. The reservoir with different percentage of PEG 6000/8000 and different pH values (Tris-HCl) was prepared. To the crystallographic 24-wells plates (Huntington Research) 980 µl of reservoir was poured in each well. On a siliconized cover glass 1.5 µl of protein and 1.5 µl of reservoir solutions were mixed. The well, of which the edge was previously also greased, was covered with the glass in such way that the drop was hanging above the reservoir solution in the well, thus rendering a closed system. Streak seeding in drops was employed to obtain compact and single crystals. Crystals which grew to appropriate size were harvested in cryo-solution containing reservoir and glycerol, and were kept in liquid nitrogen until x-ray diffraction data collection using a synchrotron.

2.2.8.2 X-ray diffraction data collection

The diffraction data were collected either at the Protein Structure Factory beamline BL14.2 of the Freie Universität Berlin at BESSY (Berliner Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung m.b.H., Berlin, Germany) or at ESRF (European Synchrotron Radiation Facility, Grenoble, France) beamline ID 14-2 at a wavelength of λ = 0,933 Å at 100 K. The

reflections were autoindexed with the program DENZO (Otwinowski and Minor, 1997), and processed and scaled with DENZO and SCALEPACK software (Otwinowski and Minor, 1997).

2.2.8.3 Structure determination and refinement

The structures of all HLA complexes were determined by molecular replacement using program PHASER (Storoni et al., 2004) with coordinates of the high-resolution crystal structures of HLA-B*2705:pVIPR (PDB code 1OGT; Hülsmeyer et al., 2004) or B*2705:β_2m:pGR (PDB code 2A83; Rückert et al., 2005) as search model (the peptide and the polymorphic residues in B*2705 as well as water molecules were omitted). After initial rigid body refinement the structures were subjected to restrained refinement using REFMAC (Murshudov et al., 1999). Electron density maps were interpreted with program O (Jones et al., 1991) and water molecules introduced with ARP/wARP (Perrakis et al., 1999). Intermediate and final structures were evaluated with PROCHECK (Laskowski et al., 1993) and WHATCHECK (Hooft et al., 1996). Figures of molecules were prepared in PyMOL (DeLano Scientific LLc, www.pymol.org).

2.2.9 Thermodynamic methods

Circular dichroism (CD) and differential scanning calorimetry (DSC) measurements were performed by Rolf Misselwitz, as recently described in detail (Hillig et al., 2004; Hülsmeyer et al., 2005). Briefly, the chromatographically purified HLA-B27:β_2m:peptide complexes were prepared in 10 mM phosphate buffer, pH 7.5, 150 mM NaCl and filtrated through 0.22 µm membrane filter (Millipore, Schwalbach, Germany). Protein concentrations were determined photometrically at 280 nm using absorption coefficients of $A_{0.1\%,\ 1cm}$ = 2.04 for B*2703:β_2m:pLMP2, 2.16 for B*2703:β_2m:pVIPR, which were calculated from the amino acid compositions (http://www.expasy.org/tools/protparam.html).

Heat-induced unfolding of complexes was performed using a JASCO 720 spectropolarimeter equipped with a Neslab temperature control system (ThermoNeslab, Portsmouth, NH, USA) at a heating rate of 20°C/h. Cuvettes with 1 mm path length and samples with 3 - 4 µM protein concentration were used. Mean residue molar ellipticities [Θ] at 218 nm were calculated with mean residue weights of 117.1 g/mol for B*2703:β_2m:pVIPR and 116.8 g/mol for B*2703:β_2m:pLMP2. The negative molar ellipticity of the complexes dropped with increasing temperature, reflecting the loss of secondary structures with unfolding. The half transition temperature T_m was determined estimating the inflection point of the ellipticity change

at 218 nm between the folded and unfolded states. Several transitions contribute to the experimental CD signal (see DSC melting). Therefore, the T_m values determined by CD do not represent the half-transition temperature of one well defined "two-state" process. Rather they indicate the temperature where just one-half was observed of the total ellipticity change.

Excessive heat capacity curves were recorded using an ultrasensitive scanning microcalorimeter (VP-DSC, MicroCal Inc., Northampton, MA, USA) at a heating rate of 1 K min^{-1} and at protein concentrations between 3 - 4 µM. To examine the reversibility of folding the samples were cooled to 20 °C after the first run and heated up a second time. The experimental curves were analyzed by standard procedures using the "ORIGIN for DSC" software package supplied by the manufacturer. The classical independent two-state model was applied for the deconvolution of all experimental excessive heat capacity curves. The model is based on the assumption that the macromolecule is composed of a number of domains, each of which is involved independently in a transition between the folded and unfolded states. T_m and ΔH_m are the thermal midpoints of transition and the calorimetric heat change determined from the area under the transition peak. The basic equations used to deconvolute the DSC data are described by Privalov and Potekhin (1986).

2.2.10 Computer analysis

BLAST, BLAST2, MEGABLAST and other programs from National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) were used for the analysis of the nucleotide sequences. Results of DNA sequencing were checked and analyzed by Vector NTI Suite 5.5 (Invitrogen) software. The analysis of protein sequences was done using ExPASy Proteomix Server (www.expasy.org). Coordinates of the proteins which structures were solved during this work will be submitted to RCSB Protein Data Bank (Research Collaboration for Structural Bioinformatics, www.rcsb.org). Figures of determined structures were prepared in PyMOL DeLano Scientific software, available on the www.pymol.org website.