2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

If not otherwise stated, all standard laboratory chemicals were of p.a. grade and were purchased from Sigma-Aldrich (Taufkirchen), Merck (Darmstadt), Carl Roth GmbH (Karlsruhe), Fischer Scientific (Loughborough, UK) or J.T.Baker (Deventer, Netherlands). The following radiochemicals were purchased from Amersham Pharmacia (Freiburg): α-[^{32}P]-dATP (10 mCi/ml), α-[^{32}P]dCTP (10 mCi/ml), α-[^{32}P]dGTP (10 mCi/ml), α-[^{32}P]dTTP (10 mCi/ml), L-[*methyl*-^{3}H]methionine (10 µCi/ml), L-[^{35}S]methionine (10 µCi/ml).

2.1.2 Enzymes and reaction kits

Enzymes

All restriction enzymes, Vent DNA polymerase, DNA polymerase I (Klenow fragment), calf intestine phosphatase, and Quick ligase were purchased from (New England Biolabs, Schwalbach). *Taq* DNA polymerase was obtained from Qiagen (Hilden). Ribonuclease A was from Sigma-Aldrich and lysozme from Eurobio (Les Ulis Cedex, France). AspN (sequencing grade) and Complete protease inhibitors were obtained from Roche (Mannheim).

Reaction kits

The indicated reaction kits were used according to the manufacturer's instructions. Any changes to the protocols are mentioned in the corresponding methods section (2.2). The QIAEX II Gel extraction Kit (500), QIAquick Nucleotide Removal Kit (250) QIAGEN® and the Plasmid Purification Kit were from Qiagen (Hilden). The Quick Ligation™ Kit was purchased from NEB. From Stratagene (La Jolla, CA) the QuickChange® II Site-Directed Mutagenesis Kit, Absolutely RNA® RT-PCR Miniprep Kit and ProSTAR™ HF Single-Tube RT-PCR System were obtained. The Half-Dye™ Mix was from Bioline (Luckenwalde), the ABI Prism BigDye™ terminator mix from Applied Biosystems (Foster City, CA), the Western Lightning™ Chemiluminescence Reagent Plus from Perkin Elmer Life Science (Norton, Ohio) and the Luciferase Assay System from Promega (Mannhein).

2.1.3 Plasmids and constructs

Plasmids and expression vectors used in this study are listed in Table 2.1.

Table 2.1. Plasmids and constructs used in this work

Plasmid	Description	Source (Reference)
pEGFP-N1	mammalian expression vector, allows N-terminal fusion to GFP	Clontech, Palo Alto, CA
pEGFP-C2	mammalian expression vector, allows C-terminal fusion to GFP	Clontech
pEGFP-C3	mammalian expression vector, allows C-terminal fusion to GFP	Clontech
pSTAT1-GFP (*)	human STAT1 in pEGFP-N1	(Begitt et al., 2000)
pSTAT1(ΔN)-GFP	STAT1(ΔN), derivative of*, lacking the N-domain	Dr. U. Vinkemeier
pN-GFP	STAT1 N-domain (aa 1-132) fused to GFP	this work
pCC-GFP	STAT1 CC-domain (aa 135-316) fused to GFP	this work
pNCC-GFP	STAT1 N-, CC-domain (aa 1-316) fused to GFP	this work
pGST-GFP	bacterial expression vector for GST-GFP fusion proteins	(Begitt et al., 2000)
pGST-NES-GFP	STAT1 NES (aa 365-427) in pGST-GFP	(Begitt et al., 2000)
pGST-NLS-GFP	SV40 NLS in pGST-GFP	(Meyer et al., 2003)
pGST-N-GFP	STAT1 N-domain (aa 1-132) in pGST-GFP	this work
pSTAT1(Δ34)-GFP	STAT1(Δ34), derivative of*, lacking aa 1-34	this work
pSTAT1(Δ44)-GFP	STAT1(Δ44), derivative of*, lacking aa 1-44	this work
pSTAT1(Δ73)-GFP	STAT1(Δ73), derivative of*, lacking aa 1-73	this work
pSTAT1(Δ96)-GFP	STAT1(Δ96), derivative of*, lacking aa 1-96	this work
pSTAT1(Δ116)-GFP	STAT1(Δ116), derivative of*, lacking aa 1-116	this work
pSTAT1(Δ45-73)-GFP	STAT1(Δ45-73), derivative of*, lacking aa 45-73	this work
pSTAT1(Δ74-96)-GFP	STAT1(Δ74-96), derivative of*, lacking aa 74-96	this work
pSTAT1(Δ97-116)-GFP	STAT1(Δ97-116), derivative of*, lacking aa 97-116	this work
pSTAT1(Δ6)-GFP	STAT1(Δ6), derivative of*, lacking aa 1-6	this work
pSTAT1(Δ11)-GFP	STAT1(Δ11), derivative of*, lacking aa 1-11	this work
pSTAT1(Δ24)-GFP	STAT1(Δ24), derivative of*, lacking aa 1-24	this work
pSTAT1(D^{11-15}A)-GFP	$D^{11}/S^{12}/K^{13}/L^{15}$A, derivative of*	this work
pSTAT1(R^{31}A)-GFP	R^{31}A, derivative of*	this work
pSTAT1(A^{31}R)-GFP	A^{31}R, derivative of*	this work
pSTAT1(R^{31}K)-GFP	R^{31}K, derivative of*	this work
pSTAT1(W^{37}A)-GFP	W^{37}A, derivative of*	(Vinkemeier et al., 1998)
pSTAT1(R^{70}A)-GFP	R^{70}A, derivative of*	this work
pSTAT1(NLS114)-GFP	$H^{81}/R^{84}/K^{85}/R^{88}$A, derivative of*	Dr. U. Vinkemeier
pSTAT1($K^{110-114}$A)-GFP	$K^{110}/E^{111}/R^{113}/K^{114}$A, derivative of*	this work
pSTAT1(E^{111}A)-GFP	E^{111}A, derivative of*	this work
pSTAT1(E^{112}A)-GFP	E^{112}A, derivative of*	this work
pGEX-5x-2	bacterial GST-expression vector	Pharmacia
pN-GST	bacterial expression of N-domain fused to the N-terminus of GST, based on pET20b	this work
pN(R^{31}A)-GST	bacterial expression of N-domain(R^{31}A) fused to the N-terminus of GST, based on pET20b	this work
pASK-IBA	bacterial expression vector allowing the C-terminal fusion to a Strep-tag	IBA, Göttingen
pASK-IBA-Stat1α	bacterial expression vector coding for STAT1 with a C-terminal Strep-tag	Dr. U. Vinkemeier

pN-Strep	bacterial expression of N-domain with a C-terminal Strep-tag	this work
pN(R^{31}A)-Strep	bacterial expression of N-domain(R^{31}A) with a C-terminal Strep-tag	this work
pET20b(+)	bacterial expression vector	Novagen, Bad Soden
pEBG	mammalian GST-expression vector	Dr. B. Mayer
pGST-STAT1-GFP	allows expression of STAT1-GFP fused to the C-terminus of GST	this work
pGST-R^{31}A-GFP	allows expression of STAT1(R^{31}A)-GFP fused to the C-terminus of GST	this work
pRcCMV Eg ΔBglII	mammalian expression vector of EpoR/gp130	(Strobl et al., 2001)
pmex/neo cd8-c-eyk	mammalian expression vector of c-eyk	(Zong et al., 1996)
pVADN-IFNAR1	mammalian expression plasmid for IFNAR1	(Ragimbeau et al., 2003)
pRc-Tyk2	mammalian expression plasmid for Tyk2	(Ragimbeau et al., 2003)
pImpα5-Strep	bacterial expression vector of importin-α5 with a C-terminal Strep-tag	Edda Schulz (AG Vinkemeier)
pSTAT1-Strep	mammalian expression of Strep-tagged STAT1	this work
pβGal	β-galactosidase expression vector	Stratagene
pGAS3xLy6E	IFNγ-responsive reporter gene vector	Wen et al., 1995a
pIRES-luc	IFNα-responsive reporter gene vector	Dr. Hengel, RKI Berlin
pNF-κB-luc	NF-κB responsive reporter gene vector	Dr. Scheidereidt, MDC Berlin
pQE-MTAP	bacterial expression vector containing the cDNA of human MTAP	Dr. D. Carson, UCSD
pGFP-MTAP	mammalian expression vector; N-terminal fusion of MTAP to GFP	this work
pGFP-NES	mammalian expression vector, N-terminal fusion of STAT1 NES (aa 365-427) to GFP	Dr. U. Vinkemeier, this work
pSG424-Crm1	mammalian expression vector, containing the human Crm1 wild type cDNA	Dr. BR. Cullen, Duke/Durham, NC
pXHCK1-hCrm1 C^{528}S	mammalian expression vector, containing the human Crm1 cDNA, with the point mutation C^{528}S	Dr. S. Miyamoto, University of Wisconsin
pFLAG	mammalian expression vector; coding for a C-terminal FLAG-epitope	this work
pCrm1-FLAG	mammalian expression vector, FLAG-tagged Crm1 (wild type)	this work
pCrm1(C^{528}S)-FLAG	mammalian expression vector, FLAG-tagged Crm1(C^{528}S)	this work
p65-GFP	mammalian expression vector; C-terminal fusion of p65 to GFP	Dr. MRH White, Liverpool
pGFP-STAT3	mammalian expression vector; N-terminal fusion of STAT3 to GFP	Dr. U. Vinkemeier

2.1.4 Primers and oligonucleotides

All oligonucleotides used for DNA sequencing, PCR- or RT-PCR amplifications and gel shift analysis were purchased from MWG-Biotech (Ebersberg) or Biotez (Berlin-Buch). The sequences of the oligonucleotides are given in Table 2.2.

Table 2.2. Primers and oligonucleotides used in this work*

Name	Sequence	Description
272	5′-ATATAAGGATCCCC**ATG**TCTCAGTGGTACGAACTTCAG-3′	N-domain fwd, BamHI
273	5′-ATATAAGAATTCTCTCTGAATATTCCCCGACTGAGCC-3′	N-domain rev, EcoRI
305	5′-ATATATGAATTC**ATG**TCTCAGTGGTACGAACTTCAG-3′	STAT1 fwd, EcoRI
306	5′-ATATATGAATTC**ATG**TCGGGGAATATTCAGAGCACAG-3′	ΔN fwd, EcoRI
353	5′-ATATATGGATCCATCATACTGTCGAATTCTAC-3′	STAT1 rev, BamHI
396	5′-CAGTCTCAACTTCACAGTG-3′	internal STAT1 primer, bp 1230 rev
430	5′-GTGGGAGGTCTATATAAGCAG-3′	internal STAT1 primer, bp 570 rev
465	5′-ACGTCGACATTTCCCGTAAATCTG-3′	(M67) 465/466
466	5′-CAGTCAGATTTACGGGAAATGTCG-3′	(M67) 465/466
611B	5′-TGCCGAAATTCAGCCGCCAGCCT-3′	internal STAT1 primer
625	5′-ATAATAGCTAGC**ATG**GAGAATAACTTCTTGCTACAGC-3′	Δ73 fwd, NheI
626	5′-ATAATAGCTAGC**ATG**GACCCAATCCAGATGTCTATG-3′	Δ96 fwd, NheI
627	5′-ATAATAGCTAGC**ATG**GAAAACGCCCAGAGATTTAATC-3′	Δ116 fwd, NheI
673	5′-ATATATGGATCCAGG**ATG**TCTCAGTGGTACGAAC-3′	N-domain fwd, BamHI
674	5′-ATATATGAATTC**GCT**CTGAATATTCCCCGACTGAG-3	N-domain rev, S^{132}, EcoRI
679	5′-CTGTCTGAAGGAAGCAAGGAAAATTCTG-3′	$E^{112}A$ fwd
680	5′-CAGAATTTTCCTTGCTTCCTTCAGACAG-3′	$E^{112}A$ rev
681	5′-GTTTTCCCATGGAAATCAAACAGTACCTGGCACAGTG-3′	$R^{31}K$ fwd
682	5′-CACTGTGCCAGGTACTGTTTGATTTCCATGGGAAAAC-3′	$R^{31}K$ rev
694	5′-GATCGGGAAAGGGAAACCGAAACTGAA-3′	(ISRE) 694/695
695	5′-GATCTTCAGTTTCGGTTTCCCTTTCCC-3′	(ISRE) 694/695
740	5′-ATATATGGATCCAGATAAAGATGTGAATGAG-3′	NES-GFP fwd, BamHI
741	5′-CGCCCCGACACCCGCCAACACCC-3′	NES-GFP rev
753	5′-GTTTTCCCATGGAAATCGCACAGTACCTGGCACAGTG-3′	$R^{31}A$ fwd
754	5′-CACTGTGCCAGGTACTGTGCGATTTCCATGGGAAAAC-3′	$R^{31}A$ rev
gB2	5′-ATAATAGAATTCCTTTTACTGTATTTCTCTC-3′	rev, EcoRI
tm001	5′-ATAATAGCTAGC**ATG**GCACAGTGGTTAGAAAAGCAAGAC-3′	Δ34 fwd, NheI
tm002	5′-ATAATAGCTAGC**ATG**CACGCTGCCAATGATGTTTC-3′	Δ44 fwd, NheI
tm019	5′-GTTATTCTCCTCCCAGTCTTGCTTTTCTAACC-3′	Δ45-73; aa 44 rev with aa 74 overhang
tm020	5′-GACTGGGAGGAGAATAACTTCTTGCTACAGC-3′	Δ45-73; aa 74 fwd with aa 44 overhang
tm021	5′-GATTGGGTCCAAAGAAAAGCGACTATATTG-3′	Δ74-96; aa 73 rev with aa 97 overhang
tm022	5′-GCTTTTCTTTGGACCCAATCCAGATGTCTATGATC-3′	Δ74-96; aa 97 fwd with aa 73 overhang
tm023	5′-GGCGTTTTCTTCCTGAAAATTATCCTGAAG-3′	Δ97-116; aa 96 rev with aa 116 overhang
tm024	5′-CAGGAAGAAAACGCCCAGAGATTTAATCAGGC-3′	Δ97-116; aa116 fwd with aa 96 overhang
tm040	5′-AATAATACATATGTCTCAGTGGTACGAACTTCAGC-3′	N-domain 5', NdeI
tm041	5′-TCCAACAGATGCACGACGATTCCCCGACTGAGCCTG-3′	N-domain/PKA 3'
tm044	5′-ATCGAAGGTCGTGGGATGTCCCCTATACTAGGTTATTGG-3′	5' PKA/GST
tm045	5′-ATTATAGAATTCACAGATCCGATTTTGGAGGATGGTC-3′	3' GST-stop, EcoRI
tm046	5′-ACCTTCGATTCCAACAGATGCACGACG-3′	N-domain/PKA 3', Xa overlap primer
tm047	5′-TCTGTTGGAATCGAAGGTCGTGGGATG-3′	5' PKA/GST, Xa overlap primer
tm051	5′-ATATATGAATTC**ATG**TCTCAGTGGTACGAACTTCAGC-3′	STAT1 (1-25), EcoRI
tm053	5′-ATATATGGATCCCCCACGACCTTCGATTCCAACAG-3′	N-domain rev, incl. Xa, PKA overlap, BamHI
tm054	5′-ATTATAGGATCC**ATG**CCAGCAATTATGACAATG-3′	hCrm1 fwd, BamHI
tm055	5′-ATATTAGGATCCCATCACACATTTCTTCTGG-3′	hCrm1 rev in frame with FLAG, BamHI
tm058	5′-GAATGGTACAGAGTGGTC-3′	sequencing primer, hCrm1 ($C^{528}S$)
tm059	5′-ATAATAGCTAGC**ATG**CTTCAGCAGCTTGACTC-3′	Δ6 fwd, NheI
tm060	5′-ATAATAGCTAGC**ATG**TCAAAATTCCTGGAGCAGG-3′	Δ11 fwd, NheI
tm061	5′-ATAATAGCTAGC**ATG**AGTTTTCCCATGGAAATC-3′	Δ24 fwd, NheI
tm066	5′-TATTAAGGATCC**GA**GCTCTGAATGAGCTGCTG-3′	CCD in pEGFP-N1, rev, BamHII, S^{316}
tm067	5′-TATATAGAATTC**ATG**TTAGACAAACAGAAAGAGC-3′	CCD fwd, EcoRI, M^{135}
tm069	5′-GGATGATCAATATAGTGCCTTTTCTTTGGAGAATAAC-3′	$R^{70}A$ fwd
tm070	5′-GTTATTCTCCAAAGAAAAGGCACTATATTGATCATCC-3′	$R^{70}A$ rev
tm082	5′-TTCAGCAGCTTGACGCAAAATTCGCGGAGCAGGTTCACCAGC-3′	$S^{12}/L^{15}AA$ fwd
tm083	5′-GCTGGTGAACCTGCTCCGCGAATTTTGCGTCAAGCTGCTGAAG-3′	$S^{12}/L^{15}AA$ rev
tm090	5′-CGAACTTCAGCAGCTTGCCGCAGCATTCGCGGAGCAGGTTCACCAGC-3′	$D^{11}/K^{13}AA$, fwd on $S^{12}/L^{15}AA$
tm091	5′-GCTGGTGAACCTGCTCCGCGAATGCTGCGGCAAGCTGCTGAAGTTCG-3′	$D^{11}/K^{13}AA$, rev on $S^{12}/L^{15}AA$
tm102	5′-GTTTTCCCATGGAAATCCGCCAGTACCTGGCACAGTG-3′	$A^{31}R$ fwd
tm103	5′-CACTGTGCCAGGTACTGGCGGATTTCCATGGGAAAAC-3′	$A^{31}R$ rev
tm110	5′-ACCGCCGTGAAGATTGGAATAA-3′	human MTAP, fwd
tm111	5′-GCCCGGGAGCTAAAACGAGGTC-3′	human MTAP, rev
B70	5′-CAGCTGTCTGAAGGCAGAAAGGAAAATTC-3′	$E^{111}A$ fwd
B71	5′-GAATTTTCCTTTCTGCCTTCAGACAGCTG-3′	$E^{111}A$ rev
B80	5′-CATTTACAGCTGTCTGGCGGCAGAAAGGAAAATTC-3′	$K^{110}A$ fwd, on $E^{111}A$
B81	5′-GAATTTTCCTTTCTGCCGCCAGACAGCTGTAAATG-3′	$K^{110}A$ rev, on $E^{111}A$
B82	5′-CTGTCTGGCGGCAGAAGCGGCAATTCTGGAAAACGCCC-3′	$R^{113}/K^{114}AA$ fwd, on $K^{110}A$, $E^{111}A$
B83	5′-GGGCGTTTTCCAGAATTGCCGCTTCTGCCGCCAGACAG-3′	$R^{113}/K^{114}AA$ rev, on $K^{110}A$, $E^{111}A$
B173	5′-CGGGCGCGGTCGGTAAAGTTGTTC-3′	*Photinus pyralis* luciferase, fwd
B174	5′-CCGCGTCGAAGATGTTGGGGTGTTGT-3′	*Photinus pyralis* luciferase, rev

*Restriction sites in the corresponding primers are highlighted in light grey, start and stop codons are given in bold; mutated codons in primers used for mutagenesis are underlined; fwd = forward primer; rev = reverse primer.

2.1.5 Bacterial strains and media

Bacteria (Table 2.3) were grown in LB medium or SOC medium with the required additives. The composition of the media is given for one liter of medium. The pH of the media was adjusted to 7.0 with HCl.

Table 2.3. *E.coli* strains used in this study

E. coli strain	Genotype	Source
BL21 (DE3) pLysS	pLysS, F^-, *ompT hsdS*$_B$($r_B^-m_B^-$) *gal dcm* λ(DE3) [pLysS Cam^R]	(Novagen)
DH5α	F- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 g*yr*A96 *rel*A1 λ-	(Gibco-BRL)
Max Efficiency®	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74	(Invitrogen)
DH10Bac™	*rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ- *rps*L	
Competent Cells	*nup*G [pMON7124 (*bom*+, *tra*-, *mob*-)]	
SG13009 [pREP4]	K12 derivative, *Nal*^{s} *Str*^{s} *Rif*^{s} *Thi*$^-$ *Lac*$^-$ *Ara*$^-$ *Gal*$^+$ *Mtl*$^-$ *F*$^-$ *RecA*$^+$ *Uvr*$^+$ *Lon*$^+$ [pREP4 Kan^R]	(Qiagen)
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*q*Z.M15* Tn*10* (Tet^R) Amy Cam^R]	(Stratagene)
XL10-Gold®	Tetr. (*mcrA*)*183* .(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44*	(Stratagene)
Ultracompetent Cells	*thi-1 recA1 gyrA96 relA1 lac* Hte [F' proAB *lacI*q*Z .M15* Tn*10* (Tet^R) Amy Cam^R]	

LB medium
10 g tryptone
5 g yeast extract
5 g NaCl

SOC medium
20 g peptone
5 g yeast extract
0.5 g NaCl
186 mg KCl
0.5% (w/v) Glucose

For preparation of solid media 15 g/l agar was added to the medium. When appropriate, antibiotics or other supplements were added at the following concentrations: 100 µg/ml ampicillin (Eurobio; Les Ulis Cedex, France), 1 mM anhydrotetracycline hydrochloride (Acros Organics; Geel, Belgium), 34 µg/ml chloramphenicol (Eurobio), 1 mM IPTG (Roth), 50 µg/ml kanamycin A (Sigma), 20 µg/ml tetracycline hydrochloride (Eurobio) or 40 µg/ml X-Gal (Roth).

2.1.6 Cell lines and cell culture reagents

The following mammalian cell lines were used in this work:

Table 2.4. Cell lines used in this study

Name	Species	Description	Source
2fTGH	human	fibrosarcoma, parental cell line of U3A	Dr. G. Stark, Lerner Research Institute, CL
U3A	human	fibrosarcoma, STAT1 deficient	Dr. G. Stark, Lerner Research Institute, CL
U3AWT-Strep	Human	stable cell line, derived from U3A reconstituted with STAT1-Strep	this work
HeLa	human	cervix carcinoma	ATCC: CCL-2, Dr. T. Lindl, IAZ
HeLa S3	human	cervix carcinoma	ATCC: CCL-2.2, DSMZ (ACC 161)
Hek, 293	human	kidney	ATCC: CRL-1573, Dr. T. Lindl, IAZ
3T3-Swiss albino	mouse	embryonic fibroblasts	ATCC: CCL-92
A431	human	epidermoid carcinoma	ATCC: CRL-1555, DSMZ (ACC 91)
COS-1	monkey, african green	kidney, SV40 transformed	ATCC: CRL-1650
COS-7	monkey, african green	kidney, SV40 transformed	ATCC: CRL-1651
Hep G2	human	hepatocellular carcinoma	ATCC: HB-8065
MCF-7	human	mammary gland adenocarcinoma	ATCC: HTB-22, Dr. Erdos

All tissue culture reagents if not otherwise stated were purchased from Gibco-BRL (Karlsruhe) or PAA Laboratories (Pasching, Austria). Culture dishes and plastic ware were obtained from the suppliers TPP AG (Trasadingen, Switzerland) or WCP (Berlin).

2.1.7 Antibodies

Primary antibodies used in this work are listed in Table 2.5.

Table 2.5. Primary antibodies used in this work

Antibody	Species	Supplier
α-STAT1 p84/p91 (E-23) sc-346	rabbit, polyclonal	Santa Cruz Biotechnology (Santa Cruz, CA)
α-STAT1 p84/p91 (C-24) sc-345	rabbit, polyclonal	Santa Cruz Biotechnology
α-STAT1 p84/p91 (C-136) sc-464	mouse, monoclonal	Santa Cruz Biotechnology

Antibody	Species	Supplier
α-Phospho-STAT1 (Y701)	rabbit, polyclonal	Cell Signaling Technology (Beverly, MA)
α-Phospho-STAT1 (S727)	rabbit, polyclonal	Upstate (New York)
α-STAT1 (N-terminus) G16920	mouse, monoclonal	Transduction Laboratories (Heidelberg)
α-Arginine (mono-, di-methyl) ab412 [7E6]	mouse, monoclonal	Abcam Limited (Cambridge, UK)
α-PIAS1 (c-20) sc-8152	goat, polyclonal	Santa Cruz Biotechnology
α-PIAS 1/3 (N-18) sc-8153	goat, polyclonal	Santa Cruz Biotechnology
α-NFκB p65 (A) sc-109	rabbit, polyclonal	Santa Cruz Biotechnology
α-CRM1 (C-20) sc-7825	goat, polyclonal	Santa Cruz Biotechnology
α-p38 MAP Kinase	rabbit, polyclonal	Cell Signaling Technology
α-Phospho-p38 MAP Kinase, (T180/Y182)	rabbit, polyclonal	Cell Signaling Technology
α-p53 (DO-1) sc-126	mouse, monoclonal	Santa Cruz Biotechnology
α-ß-Actin (AC-15)	mouse, monoclonal	Sigma
α-Eps15 (C-20) sc-534	rabbit, polyclonal	Santa Cruz Biotechnology
α-Sp1 (1C6)sc-420	mouse, monoclonal	Santa Cruz Biotechnology
α-GST	goat, polyclonal	Pharmacia Biotech
α-HA High Affinity (3F10)	rat, monoclonal	Roche
α-FLAG® M2	mouse, monoclonal	Sigma
α-His-tag	rabbbit, polyclonal	Cell Signaling Technology
α-GFP	polyclonal rabbit	Dr. Rosenthal, FMP
α-Strep-tag II	polyclonal rabbit	IBA

Secondary antibodies and fluorescence probes

All peroxidase-conjugated secondary antibodies used for detection in Western blotting (goat anti-mouse IgG, swine anti-rabbit IgG, rabbit anti-goat IgG) were purchased from DAKO Cytomation (Glostrup, Denmark). Cy™ 3-conjugated secondary antibodies (goat anti-rabbit IgG, donkey anti-mouse IgG, donkey anti-goat IgG were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Tetramethylrhodamine isothiocyanate (TRITC)-coupled BSA (0.2 µg/µl) used as injection marker was obtained from Sigma-Aldrich.

2.1.8 Affinity matrices

The following affinity matrices were used: Protein A agarose (Repligen; Waltham, CA), Protein A/G agarose (Santa Cruz Biotechnology), Strep®Tactin sepharose (IBA), M2 agarose beads (Sigma-Aldrich), glutathione sepharose beads (Amersham Bioscience).

2.1.9 Peptides

Peptides used in this study were purchased from Biosynthan (Berlin-Buch).

2.1.10 Buffers and solutions

All aqueous solutions were prepared with de-ionised water (Milli-Q, UF Plus Millipore; Bedford, MA) and if not otherwise stated autoclaved at 120°C for 20 min. Buffer compositions are given in the corresponding methods section.

2.1.11 Software

GeneTool (BioTools; Edmonton, Canada) was used for the design of primers, EasyWin32 (Herolab GMBH, Wiesloch) for the analysis of DNA fragments. For the recording and archivation of pictures obtained by flouresence microscopy the programm Axiovision 3.0 (Zeiss, Oberkochen) was used. Western blots were quantified using the LumiAnalyst software (Roche Diagnostics). For the analysis of reporter gene assays the programm Prism3 (GraphPad Software; San Diego, CA) was used. The ImageQuant software (Amersham Bioscience, Freiburg) was employed for quantification of gel shifts. The web-based programm ClustalW 1.8 (www.searchlauncher.bcm.tmc.edu/multi-align/multi-align.html) was used for sequence alignments. The alignments were highlighted with BOXSHADE 3.21 (www.ch.embnet.org/software/BOX_form.html) or Weblogo (Crooks et al., 2004; weblogo.berkeley.edu/). For family tree analysis MEGA 2.1 (Kumar et al., 2001) was used. MolMol 2K.2 (Koradi et al., 1996) served the graphical depiction of protein structures. FindMod (available on the internet at www.expasy.ch/tools/findmod) was used to interpret the MS spectra.

2.2.Methods

2.2.1 Molecular cloning techniques

Standard molecular cloning methods were performed according to Sambrook and Russell (2001).

2.2.1.1 Polymerase chain reaction (PCR)

PCR fragments were generated using Vent polymerase® (NEB). For each reaction 1.25 U polymerase, 50 ng template DNA, 125 ng of each primer and 6.25 mM of each dNTP were used in a total volume of 50 µl 1x reaction buffer. PCR was performed with a Mastercycler gradient (Eppendorf, Hamburg) or a GeneAmp® PCR System 9700 (PE Applied Bioscience, Darmstadt). Following an initial denaturation step at 94°C, 18 cycles with the following parameters were performed: denaturation for 25 sec at 94°C, annealing for 30 sec, and elongation at 72°C. The annealing temperature was adjusted to the calculated melting temperature [T_m = 64.9°C + 41°C x (G+C - 16.4)/N, where N = total number of bases] of the respective primer pair. Elongation time was 1 minute per kb to be amplified. Five µl of a PCR product were checked on an analytical agarose gel. Before restriction digestion, primer and polymerase were removed with the QIAquick Nucleotide Removal Kit (Qiagen).

2.2.1.2 Restriction digestion of DNA

All restriction enzymes were purchased from NEB Biolabs. For analytical preparation 1 µg of small scale preparation DNA (2.2.1.7) was used in a digestion reaction according to the manufacturer's instructions. For preparative purposes 0.5 µg vector DNA or 35 µl PCR product (2.2.1.1) were used in a total volume of 50 µl. If necessary, vector DNA was treated for 30 min at 37°C with 0.5 µl CIP (calf intestinal phosphatase; 10 U/µl, NEB) to prevent religation of the vector.

2.2.1.3 DNA gel electrophoresis

Horizontal agarose gels (0.5-1.5%) were used to separate DNA fragments. For analytical gels agarose NEEO ultra quality (Roth) was used and dissolved in 1x TAE supplemented with 0.3 µg/ml ethidium bromide (Eurobio). Electrophoresis was performed with 1x TAE at 80-100 V using the separation system B1, B1A (Owl; Portsmouth, NH) and the electrophoresis power supply, EPS 301 (Amersham). The DNA Molecular Weight Marker X

(Boehringer) was used as a reference for size determination of the DNA fragments. DNA bands were detected with UV light (260 nm), using a transilluminator (Herolab).

TAE (50x)
2 M Tris-Acetate
1 M NaAc
0.05 M EDTA
adjust pH with HAc to 8.3

DNA sample buffer (10x)
TAE (10x)
30% (v/v) Glycerol
0.25% (w/v) Bromphenol blue

Low melting point agarose (Invitrogen, Karlsruhe) was used for preparative gels. The band of interest was excised from the gel with a sterile scalpel and purified with the QIAEX II Gel extraction Kit (Qiagen).

2.2.1.4 Ligation

Ligation reactions were performed in a total volume of 20 µl using the Quick Ligation™ Kit (NEB) according to the supplied instructions. For transformation of the *E.coli* strain DH5α 1.5 µl of a ligation reaction were used.

2.2.1.5 Preparation of competent bacteria

If not otherwise stated, competent bacteria were obtained with the $CaCl_2$ method (Cohen et al., 1972). One hundred ml LB medium (2.1.5) were inocculated with 200 µl of an overnight culture of the corresponding *E.coli* strain. The culture was incubated 2-3 h hours at 37°C with vigorous shaking to an optical density at a wavelength of 600 nm (OD_{600}) of ~0.6. Subsequently, the culture was centrifuged for 10 min at 4,000 x g at 4°C (centrifuge 5804R, Eppendorf), and the supernatant was discarded. The pellet was resuspended in 30 ml ice-cold, sterile-filtered 100 mM $CaCl_2$, and incubated for 1 h on ice. Following re-centrifugation, the pellet was resuspended in 1.3 ml ice-cold 100 mM $CaCl_2$, and 0.7 ml glycerol. Aliquots of the bacteria suspension (100 µl) were quick-frozen in liquid nitrogen, and stored at -80°C.

2.2.1.6 Transformation of bacteria

Plasmid DNA was introduced into bacteria via the heatshock method (Cohen et al., 1972). One hundred µl competent bacteria were carefully thawed on ice and mixed with 10 ng plasmid DNA or 1.5 µl of a ligation reaction (2.2.1.4). Following 30 min incubation on ice with occasional shaking, the mixture was incubated for 45 sec at 42°C in a water bath and subsequently cooled on ice for 5 min. After addition of 600 µl LB medium the cells were incubated for 1 hour at 37°C with vigorous shaking to express the antibiotic resistance

conveyed by the plasmid. Three hundred µl of the bacteria suspension were plated on an agar plate containing the corresponding antibiotic and incubated overnight at 37°C.

2.2.1.7 Isolation of plasmid DNA from bacteria

For amplification of plasmid DNA the bacterial strain DH5α was transformed. Plasmids were isolated from overnight cultures inocculated with a single colony. Cultures were grown at 37°C in LB medium containing the appropriate antibiotic.

Small scale isolation of plasmid DNA

From an overnight culture 1.5 ml were transferred to a snap vial (Eppendorf). After centrifugation for 1 min at RT with maximum speed (16,000 x g; table centrifuge 5415D, Eppendorf) the pellet was resuspended in 150 µl TELT buffer supplemented with 10 µl lysozym (25 µg/µl, Eurobio). After 5 min incubation at RT, 5 min boiling at 95°C and 5 min chilling on ice the lysate was centrifugated at 16,000 x g at 4°C (table centrifuge 5415R, Eppendorf) for 8 min. The pellet was discarded and the plasmid DNA in the supernatant was precipitated by adding 120 µl of isopropanol. Following 15 min centrifugation at 16,000 x g at 4°C, the supernatant was discarded. The pellet was washed by the addition of 250 µl 70% (v/v) EtOH and air-dried for 5-10 min. Finally, the pellet was resuspended in 20 µl TE buffer including 0.5 µg/µl RNase A (Sigma). One µl small scale preparation DNA (~1 µg) was used in an analytical restriction digest. DNA samples were stored at -20°C.

TELT
2.5 ml 1 M Tris-HCl, pH 7.5
39.3 ml 3.2 M LiCl
6.25 ml 0.5 M EDTA
2 ml 10 % (v/v) Triton X-100
add H_2O to 100 ml

TE
10 mM Tris-HCl, pH 8.0
1 mM EDTA

Large scale isolation of plasmid DNA

For large scale purification of plasmid DNA, 100 ml of an overnight culture containing the appropriate antibiotic were used. The purification was performed using the QIAGEN® Plasmid Purification Kit (Qiagen) following the manufacturer's instructions. The DNA concentration was determined by measuring the optical density of the sample at a wavelength of 260 nm (OD_{260}) using a Ultrospec 2100pro photometer (Amersham, Freiburg) and a quartz cuvette (Hellma, Jena). An OD_{260} of 1 at a path length of 1 cm corresponded to a DNA concentration of 50 µg/ml.

2.2.1.8 DNA sequencing

Verification of DNA sequences was performed using the chain termination method by Sanger et al. (1977). A total of 300 ng purified plasmid DNA or 1 µl of small scale preparation DNA (2.2.1.7) were used as template in the PCR. The reaction was carried out with 5-10 pmol sequencing primer, 2.5 µl BigDye™ terminator mix and 5 µl Half-Dye™ Mix (Bioline) in a total volume of 20 µl. Cycling parameters were 30 cycles of: 16 sec at 94°C denaturation, 16 sec at 52°C annealing and 2 min at 60°C elongation. Following amplification, the PCR products were precipitated by addition of 2 µl of buffer containing 1.5 M NaAc, 250 mM EDTA, pH 8.5 (NaOH), and 80 µl 95% (v/v) EtOH. The mixture was incubated for 15 min on ice and centrifuged for 15 min at 4°C and 16,000 x g. Subsequently, the pellet was washed with 80% (v/v) EtOH and air-dried. Sequence analysis was done on an ABI Prism^{TM} capillary sequencer by the DNA sequencing service group of the FMP.

2.2.1.9 Site-directed mutagenesis

Point mutations were introduced into the cDNA of STAT1 and CRM1 by using the QuickChange® II Site-Directed Mutagenesis Kit (Stratagene). A total of 50 ng of the parental plasmid DNA was used for PCR. The reaction was performed in a 50 µl volume containing 125 ng of each primer carrying the mutation, 1 µl dNTPs (dATP, dCTP, dGTP, and dTTP; 1.25 mM each), 2.5 U Pfu Turbo® polymerase (Stratagene) and 5 µl of 10x Pfu polymerase buffer (Stratagene). The cycling parameters were 14 cycles of 20 sec denaturing at 94°C, 20 sec annealing at 52°C, and 2 min/kbp elongation at 68°C. The amplified plasmids were then digested with 1 µl of DnpI for 1 h to remove the methylated parental DNA of bacterial origin. An aliquot of the digest reaction (1.5 µl) was used to transform XL10-Gold Ultracompetent *E. coli* Cells (Stratagene). Plasmids obtained after small scale preparation (2.2.1.7) were verified by DNA sequencing for the presence of the point mutation.

2.2.1.10 Plasmid construction

Plasmids constructed in this work are described in Table 2.1. pSTAT1-GFP has been described (Begitt et al., 2000).

pSTAT1(ΔN)-GFP: Deletion of the aminoterminal 126 residues of STAT1 was done by PCR with the primer pair 306/353. The resulting product was digested with EcoRI and SmaI and cloned into pEGFP-N1. In a second step the sequence was completed by adding the SmaI/NotI fragment from pSTAT1-GFP. The final construct was named pSTAT1(ΔN)-GFP.

N-terminal truncation mutants were constructed by replacing the NheI/SpeI fragment of pSTAT1-GFP via shortened PCR-fragments digested with the same enzymes. The primer pair used for PCR-amplification is given in brackets. pSTAT1(Δ34)-GFP, (tm001/611B); pSTAT1(Δ44)-GFP, (tm002/611B); pSTAT1(Δ73)-GFP, (625/gB2); pSTAT1(Δ96)-GFP, (626/611B); pSTAT1(Δ116)-GFP, (627/611B); pSTAT1(Δ6)-GFP, (tm059/396); pSTAT1(Δ11)-GFP, (tm060/396); pSTAT1(Δ24)-GFP, (tm061/396).

N-terminal internal deletions mutants were generated in two consecutive rounds of PCR. In a first round the coding regions flanking the deletion site were amplified separately and in a second round of PCR the two fragments were "ligated" by means of sequence overlaps. The resulting PCR product was inserted into the Nhe/SpeI site of pSTAT1-GFP. pSTAT1(Δ45-73)-GFP, (tm019/430, tm020/gB2); pSTAT1(Δ74-96)-GFP, (tm021/430, tm022/gB2); pSTAT1(Δ97-116)-GFP, (tm023/430, tm024/gB2).

pN-GFP, pCC-GFP, pNCC-GFP: A mammalian expression vector encoding the N-domain of STAT1 fused to the N-terminus of GFP was prepared as follows: The coding sequence of the N-domain (aa 1-132) was PCR amplified (673/674), digested with BamHI/EcoRI and inserted into pEGFP-N1 digested with BglII/EcoRI. The resulting construct was named pN-GFP. A fusion of the coiled-coil domain (CC, aa 135-316) or the N-domain with the coiled-coil domain (NCC, aa 1-316) to GFP was achieved by amplifying the corresponding cDNA regions with the primer pairs tm066/tm067 and 305/tm066, respectively. The digested PCR products were inserted into the pEGFP-N1 vector digested with EcoRI/BamHI.

pGST-N-GFP: To allow expression as a GST-GFP fusion protein, the N-domain coding sequence (aa 1-131) was PCR-amplified with the primer pair 272/273. The resulting PCR-product was digested with EcoRI and BamHI and inserted into pGST-GFP.

Point mutations in the coding sequence of pSTAT1-GFP were introduced by site-directed mutagenesis (Quick-Change Kit, Stratagene). The amino acid substitution and the primers used are given in brackets. pSTAT1($D^{11-15}A$)-GFP harbours the following amino acid substitutions: $D^{11}/S^{12}/K^{13}/L^{15}A$ (tm082/tm083, tm090/tm091); pSTAT1($R^{31}A$)-GFP (753/754); pSTAT1($R^{31}K$)-GFP (681/682); pSTAT1($A^{31}R$)-GFP (tm102/tm103); pSTAT1($R^{70}A$)-GFP (tm069/tm070); pSTAT1($E^{111}A$)-GFP (B70/B71); pSTAT1($E^{112}A$)-GFP (679/680); In the coding sequence of pSTAT1($K^{110-114}A$)-GFP the codons for $K^{110}/E^{111}/R^{113}/K^{114}$ were changed to alanine (B70/B71, B80/B81, B82/B83). Mutations were verified by DNA sequencing.

pGST-STAT1-GFP, pGST-R^{31}A-GFP: Fusion of STAT1 to the C terminus of GST was accomplished by cloning the NotI fragment of pSTAT1-GFP containing STAT1-GFP into the NotI site of the mammalian GST fusion vector pEBG (a kind gift of Dr. B. Mayer, University of Connecticut). The insertion of the NotI fragment from the pSTAT1(R^{31}A)-GFP into pEBG resulted in pGST-R^{31}A-GFP.

pN-GST, pN-Strep: The N-domain of human STAT1 was expressed in bacteria as an N-terminal fusion to GST (N-GST) or the Strep-tag (N-Strep). For generation of N-GST, a cDNA stretch encoding aa 1-129 of STAT1 was amplified by PCR with the primer pair tm040/tm041. The cDNA of GST was amplified from pGEX-5X-2 with the primer pair tm044/tm045. The N domain reverse primer and the GST forward primer additionally encoded non-overlapping parts of factor Xa and protein kinase A (PKA) recognition sites. In a second step the resulting PCR product representing the N domain was re-amplified with the primers tm040/tm046; the GST cDNA fragment was re-amplified with the primer pair tm045/tm047. The resulting products allowed for PCR-mediated ligation in a third round of PCR (tm040/tm045). The final PCR product was cut with NdeI and EcoRI and inserted into pET20b(+) (Novagen, Bad Soden). The resulting vector encoded the N domain connected to GST via a linker consisting of the PKA recognition sequence and the Xa cleavage site. Alternatively, the N domain was expressed in bacteria with a Strep-tag at its C terminus. The respective expression vector was prepared by PCR amplification of the N domain cDNA including PKA and factor Xa recognition sites from N-GST in pET20b(+) with the primer tm051/tm053. The PCR product was cut with EcoRI and BamHI and introduced into pASK-IBA3. PCR was performed with Vent proofreading enzyme. The correct sequence of the constructs was confirmed by DNA sequencing.

pSTAT1-Strep: a eukaryotic expression vector coding for STAT1 with a C-terminal Strep tag was constructed in several steps. First, a fragment coding for the Strep tag (containing a C-terminal stop codon) linked to the STAT1 C terminus was released by HindIII digestion from the bacterial expression vector pASK-IBA-Stat1α (Dr. Uwe Vinkemeier). Subsequently, the fragment was inserted into the appropriately prepared (after removing the STAT1 coding region by digestion with EcoRI, religation, and digestion with HindIII) empty vector pSTAT1-Flag (Meyer et al. 2002a). Finally, the STAT1 sequence was completed by introducing the BglII/XbaI fragment from pSTAT1-GFP. The resulting vector pSTAT1-Strep is based on a pEGFP backbone and expresses human STAT1 (aa 1-749) with a Strep tag at the C terminus followed by a stop codon.

pGFP-MTAP: A eukaryotic GFP-MTAP expression construct was prepared from pQE-MTAP (a kind gift of Dr. D. Carson, UCSD). The cDNA was cut out with PstI and inserted into pEGFP-C2.

pGFP-NES: A mammalian expression vector encoding a fusion protein of GFP with a STAT1-derived NES activity (aa 365-427) was constructed by PCR-amplification of the cDNA of NES-GFP from pGST-NES-GFP using the primers 740/741. After restriction digestion with the enzymes BamHI and EcoRI the resulting fragment encoding the STAT1-NES was inserted into the BglII/EcoRI sites of the vector pEGFP-C3 (Clontech) to yield pGFP-NES.

pFLAG, pCrm1-FLAG, pCrm1(C^{528}S)-FLAG: The cDNAs for wild type CRM1 and the CRM1 mutant Cys^{528} to Ser (TGT>TCT) were kindly provided by Dr. Bryan R. Cullen (Duke University, Durham) and Dr. Shigeki Miyamoto (University of Wisconsin), respectively. To allow for indirect immunodetection of recombinant CRM1, FLAG-tagged expression constructs were prepared as follows. The wild type and mutant CRM1 coding sequences were amplified by PCR using the primer pair tm054/tm055. The resulting cDNA was digested with BamHI and inserted into the BglII sites of the vector pFLAG, which was derived from pSTAT1-Flag (Meyer et al., 2002a) after removing the STAT1 coding region by digestion with EcoRI. pFLAG allows the expression of a C-terminal FLAG epitope (DYKDDDDK) followed by a stop codon. The constructs were confirmed by DNA sequencing across the mutated codons and the restriction sites used for cloning.

2.2.2 Cell culture techniques

2.2.2.1 Cultivation of mammalian cells

Cell lines used in this study are listed in Table 2.4. They were grown at 37°C in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM; Biochrom, Berlin) containing 10% (v/v) fetal calf serum (FCS; Biochrom) and 1% (w/v) penicillin/streptomycin (Biochrom). Cells were grown to a maximal density of 1-2 x 10^6 cells/cm^2 and subcultured every 2-3 days. To detach adherent cells they were washed twice with PBS (without Ca^{2+} and Mg^{2+}; Biochrom) and incubated for up to 5 min with trypsin/EDTA (Viralex™, PAA, Linz) at 37°C. Trypsination was stopped by addition of serum containing culture medium.

Freezing cells

Cells were grown to subconfluency in 6 well plates and were detached by trypsination. Subsequently, they were pelleted by centrifugation at 1000 x g for 5 min at 15°C (centrifuge 5804R, Eppendorf) and resuspended in 0.5 ml DMEM containing 20% (v/v) FCS, 10% (v/v) dimethylsulfoxide (DMSO). The cell suspension was transferred to cryo-vials which were stored in a cryo freezing container (Nalgene; Neerijse, Belgium), filled with isopropanol. Following incubation for 1 hour at 4°C, and overnight at -80°C the cells were stored in liquid nitrogen.

Thawing cells

Frozen cells were quickly thawed at 37°C in a water bath. The cells were pelleted at 1000 x g for 5 min and resuspended in DMEM containing 20% (v/v) FCS and subsequently seeded in fresh culture medium at a density of 1 x 10^6 cells/cm^2. Culture medium was replaced the following day to remove residual DMSO.

2.2.2.2 Transfection and generation of stable cell lines

Transient transfections were performed by using Lipofectamine-plus (Gibco-BRL) according to the manufacturer's instructions. Routinely, the following amounts of plasmid DNA were used: 5 µg per 10 cm dish, 2 µg per 3.5 cm dish, and 0.4 µg per 12-well. Following transfection, cells were cultivated for another 24-48 hours before analysis. U3A cells stably expressing STAT1-Strep were generated by clonal selection in the presence of 0.65 mg/ml G418 (Calbiochem; San Diego, CA). A 10 cm dish U3A cells was transfected with 5 µg pSTAT1-Strep (2.2.1.10). Twenty-four hours after transfection, the cells were trypsinised and diluted 1:50 to 1:100 in selection medium containing the antibiotic. The selection medium was changed every three days. Approximately 2-3 weeks later, single colonies were picked with sterile toothpicks and expanded. STAT1-containing clones were identified in a Western blot analysis (2.2.7) and maintained in selection medium.

2.2.2.3 Treatment of cells

Treatment of cells was with 5 ng/ml human IFNγ (Biomol, Hamburg), 500 IU/ml human IFNα (Biomol), 20 ng/ml human TNFα (Strathmann, Hannover) or 500 nM staurosporine (Sigma). 5'-Deoxy-5'-methylthioadenosine (MTA, Sigma) was dissolved in DMSO (100 mM/ml) and added to the cells at a final concentration of 0.3 - 0.75 mM starting 3 h before and during stimulation with IFN. DMSO (0.75% final) was added to control cells.

Leptomycin B (LMB, Sigma) and ratjadone A (RAT, a kind gift of Dr. Markus Kalesse, Universität Hannover) were dissolved in methanol (10 ng/µl) and both used in culture medium at a final concentration of 10 ng/ml.

2.2.3 Microinjections

For microinjection cells grown on coverslips were transferred into 3.5 cm dishes filled with 1 ml culture medium. The recombinant proteins (1 µg/µl) in microinjection buffer were injected into the nucleus of HeLa S3 cells using a Transjector 5246 (Eppendorf). They were co-injected with TRITC-coupled BSA (0.2 µg/µl; Sigma) to indicate the site of injection. Approximately fifty cells were injected within 15 min. After injection, the cells were incubated at 37°C for another hour before fixation in 4% (w/v) formaldehyde in PBS for 15 min. Nuclei were stained with Hoechst 33258 (5 µg/ml in PBS; Sigma) for 3 min. After washing three times with PBS and once with H_2O, the cells were mounted in Dako fluorescence mounting medium (DakoCytomation).

Microinjection buffer
20 mM HEPES-OH, pH 7.5
110 mM KAc
0.5 mM EDTA
5 mM DTT

2.2.4 Immunocytochemistry and fluorescence analysis

For microscopic analysis cells were grown on poly-L-lysine (Sigma)-coated glass coverslips in 12-well plates. Twenty-four hours after transfection, cells were stimulated for the times indicated, with subsequent fixation. Cells expressing GFP-fusion proteins were fixed for 15 min in 4% (v/v) formaldehyde/PBS. Following Hoechst staining, they were mounted as described above (2.2.3). For immunodetection of FLAG-tagged CRM1, a mouse monoclonal antibody directed against the FLAG epitope (M2; Sigma) was employed. Twenty-four hours after transfection the cells were fixed for 15 min in 4% (v/v) formaldehyde/PBS, then permeabilised for 10 min with 0.2% (v/v) Triton X-100 (Promega), and blocked for 30 min with 10% FCS (v/v) in PBS. Incubation with the α-FLAG antibody antibody at a dilution of 1:1000 was done in 10% FCS in PBS at 4°C overnight. After repeated washing in PBS, the cells were incubated for 1 h at RT with a Cy3-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) at a dilution of 1:2000 in 10% FCS in PBS. Following Hoechst staining and extensive washing in PBS, the cells were mounted as described above. Detection of endogenous STAT1 was performed using a STAT1-specific

primary antibody (E-23 or C-24, both from Santa Cruz Biotechnologies) at a dilution 1:3000 in 10% FCS in PBS for 1 hour. The secondary antibody (Cy3-conjugated goat anti-rabbit IgG) was applied for 30 min at a dilution of 1:2000 in 10% FCS in PBS. Cells were examined by conventional fluorescence microscopy using an Axioplan 2 Imaging system (Zeiss, Oberkochen). The GFP signal was detected at a wavelength of 480 nm, TRITC and Cy3 at 580 nm, and Hoechst dye at 280 nm. Images were recorded with a cooled Sensicam-CCD camera (PCO, Kehlheim).

2.2.5 Cell extraction

Preparation of whole cell extracts

For analysis of STAT1 expression and phosphorylation the cells were routinely lysed in whole cell extract (WCE) lysis buffer. This lysis buffer contains the phosphatase inhibitors vanadate and sodium fluoride and the competitive inhibitor glycerophosphate. Protease inhibitors (Complete, Boehringer Mannheim, and 0.1 mM PMSF) as well as the detergent and 1 mM dithio-1,4-threitol (DTT) were always added prior to extraction. Cells were rinsed briefly with ice-cold PBS, and subsequently they were lysed in the following volume lysis buffer: Per 10 cm culture dish 0.5 ml WCE or 0.15 ml WCE per 3.5 cm culture dish was used. The cells were scraped off the culture dish with a rubber policeman and mechanically disrupted by gently pipetting up and down. Following 30 min incubation on ice, the lysates were centrifuged at 16.000 x g for 5 min at 4°C. The supernatants were transferred to fresh tubes, and if not analysed immediately, the whole cell extracts were frozen on dry ice and stored at -80°C.

WCE buffer
50 mM Tris-HCl, pH 7.4
280 mM NaCl
0.2% (w/v) EDTA
2 mM EGTA
10% (v/v) Glycerol
50 mM NaF
10 mM Glycerophosphate
1 mM Vanadate
0.5 % (w/v) NP-40

Preparation of cytosolic and nuclear extracts

For the preparation of cytosolic extracts cells were lysed as described before except that hypotonic cytosolic extraction buffer was used. Again protease inhibitors and 1 mM DTT

were added prior to extraction. After 5 min on ice, the lysates were spun for 10 sec at 16,000 x g, 4°C to pellet the nuclei. The supernatants were aliquoted after recentrifugation for 15 min and used as cytoplasmic extracts. The pellets containing the nuclei were redissolved in an equal volume of hypertonic nuclear extraction buffer. Following 30 min extraction on ice the lysates were centrifuged for 20 min at 16,000 x g and the supernatants were used as nuclear extracts.

Cytosolic extraction buffer
20 mM HEPES-OH, pH 7.4
10 mM KCl
10% (v/v) Glycerol
1 mM EDTA
0.1 mM Vanadate
0.2 % NP-40

Nuclear extraction buffer
20 mM HEPES-OH, pH 7.6
420 mM KCl
20% (v/v) Glycerol
1 mM EDTA
0.1 mM Vanadate

2.2.6 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins or cell extracts were separated on vertical, discontinuous SDS-polyacylamide gels (Laemmli, 1970). Depending on the required separation range the acrylamide concentration was adjusted to 7%, 10%, or 12% in casting gel buffer using the rotiphorese gel®, acrylamide/bisacrylamide 37.5:1 mixture (30.8%, Roth). Shortly before casting, 0.01% (v/v) ammonium peroxosulfate (APS, Roth), and 0.01% (v/v) N,N,N',N'-Tetramethyl-ethylendiamine (TEMED, Merck) were added. After complete polymerisation, stacking gels were layered on top of the casting gel. The composition of the stacking gel was 5% acrylamide in stacking gel buffer. Samples were prepared by addition of 15% (v/v) 6x SDS sample buffer and heating at 95°C for 5 min. A microliter syringe (Hamilton; Reno, NV) was used for loading the gels. Electrophoresis was performed with SDS running buffer at 17 V/cm^2 using Protean®II electrophoresis chambers (Biorad) and the electrophoresis power supply EPS E835 (Consort; Turnhout, Belgium). The prestained molecular weight marker SeaBlue™ Plus2 (Invitrogen) was used as a size reference.

4x Casting gel buffer
1.5 M Tris-HCl, pH 8.8
0.4% (w/v) SDS

4x Stacking gel buffer
0.5 M Tris-HCl, pH 6.8
0.4% (w/v) SDS

6x SDS sample buffer
21 ml Stacking gel buffer
11.34 g Glycerol
3 g SDS
2.97 g DTT
0.01% (w/v) Bromphenol blue
add H_2O to 30 ml

10x SDS running buffer
10 g SDS
144 g Glycine
30.2 g Tris
add H_2O to 1 L
adjust pH with HCl to 8.3

2.2.7 Immunoblot analysis (Western blot)

For immunodetection of proteins, lysates were subjected to immunoblot analysis. Samples were first separated via SDS-PAGE (2.2.6) and subsequently transferred to a nitrocellulose membrane (Protan, Schleicher & Schüll, Dassel) by electroblotting using the semi-dry technique (Towbin et al., 1979). Blotting was performed at 18 V for 45 min with the Transblot®SD Semidry Transfer Cell (BioRad) and an EPS E802 power supply (Consort). Membranes were next incubated for at least 20 min in blocking solution. The primary antibody was added at a standard dilution of 1:2000 and incubated either for 30 min to 1 h at RT or ON at 4°C. Following three 5 min washing steps with TBS-T, the membranes were incubated with the horse radish peroxidase-coupled secondary antibody at a dilution 1:2000 in blocking solution for 30 min at RT. Following three washes with TBS-T and one wash with H_2O, the blot was developed using the Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer; Wellesly, MA) according to the manufacturer's instructions. The detection of the chemiluminescence was performed with a Lumi Imager system (F1, Boehringer Mannheim).

Blotting buffer (10x)
0.48 M Tris-HCl, pH 7.3
13 mM SDS
0.39 M Glycine

Transfer buffer
1x Blotting buffer
20% MeOH

TBS (10x)
12.1 g Tris-HCl, pH 7.3
80 g NaCl
add H_2O to 1 L

Blocking solution
TBS-T
4% (w/v) BSA

TBS-T
1x TBS
0.05% (v/v) Tween 20

To reprobe a blotting membrane with a different primary antibody, the membranes were stripped off the bound antibodies. They were incubated for 1 h at 50°C in stripping buffer containing 100 mM β-mercaptoethanol. Following three times washing with TBS-T for 20 min each, the membranes were blocked for at least 1 h in blocking solution before addition of the new primary antibody.

Stripping buffer
62.5 mM Tris-HCl, pH 6.8
2% (w/v) SDS
100 mM β-Mercaptoethanol

2.2.8 Coomassie staining

Detection of proteins in SDS-PAGE was possible by staining the gels with Coomassie Brilliant blue R 250 (Merck). Gels were placed for 30 min in Coomassie solution and subsequently destained by three incubations in the destaining solution for 10 min each. Alternatively, gels were destained by repeated boiling in water using a microwave.

Coomassie solution
40% MeOH
10% HAc
0.1% (w/v) Coomassie Brilliant blue R 250

Destaining solution
40% MeOH
10% HAc

2.2.9 Silver staining

Silver staining was performed using the Silver Stain Plus Kit (Bio-Rad) according to the manufacturer's instructions.

2.2.10 Electrophoretic mobility shift assay (EMSA)

DNA binding activity in cytosolic or nuclear extracts was assessed by gel shift analysis (Begitt et al., 2000). The formation of STAT1 homodimers was detected using a M67 GAS probe (465/466, Tab. 2.2), the presence of ISGF3 was with an ISRE probe (694/695, Tab. 2.2). The probes were obtained by annealing two oligonucleotides which had non-complementary 5'-GATC ends that were exploited for labelling with radioactive nucleotides.

Annealing of the oligonucleotides

One hundred pmol of each oligonucleotide were combined in 100 µl oligonucleotide buffer and denatured at 95°C for 5 min in a water bath. Annealing was achieved via slowly cooling the water bath to RT over a time of 1-2 hours. The annealed oligonucleotides were stored at -20°C.

Oligonucleotide buffer
30 mM HEPES-KOH, pH 7.4
100 mM KAc
2 mM MgAc

Labelling of the probes

Labelling was performed with the Klenow polymerase (NEB). One µl annealed oligonucleotide (1 pmol/µl), 8 µl of each radioactive nucleotide (α-[^{32}P]dATP, α-[^{32}P]dCTP, α-[^{32}P]dGTP, α-[^{32}P]dTTP (10 mCi/ml)), 5 µl 10x EcoPol buffer (NEB), and 1 µl Klenow polymerase were combined in a total volume of 50 µl. The labelling mix was incubated for 25 min at RT. Subsequently, 1 µl non-radioactive dNTPs (dATP, dCTP, dGTP, and dTTP; 6.5 mM each) were added. Five minutes thereafter, the labelling reaction was stopped by addition of 1 µl of 0.5 M EDTA. To separate the labelled probe from free radioactivity, MicroSpin™ G-25 columns (Amersham) were used according to the manufacturer's instructions. The probe was stored at 4°C.

Shift reaction

Per reaction, 4.5 µl cytosolic extract (2.2.5), 0.2 µl labelled probe, 1 µl poly-dIdC (2 mg/ml), 2.5 µl 5x shift buffer, 1.3 µl DTT (100 mM) and 3.5 µl H_2O were combined and incubated for 15 min at RT to allow complex formation. Subsequently, the reaction mix was separated on a native, 4.8% polyacrylamide gel (Fried and Crothers, 1981). For the preparation of three gels, 12 ml rotiphorese gel® [acrylamide/bisacrylamide 29:1 mixture (40%, Roth)], 4.8 ml 5x TBE, and 82 ml H_2O were combined. Polymerisation was started just before casting of the gels by the addition of 2 ml APS (10% w/v, Roth) and 100 µl TEMED (Merck). Electrophoresis was performed for 3 h at 400 V using 0.25x TBE as running buffer. After electrophoresis, the gels were placed on a Whatman paper (Schleicher & Schüll) and dried for 1 h at 80°C on a vacuum gel drier (Biometra, Göttingen). Following overnight exposure to a phosphoimager screen, the signal was detected using a phosphoimager (Storm820; Molecular Dynamics, Piscataway, NJ) and analysed using the ImageQuant software (Amersham).

5x Shift buffer
100 mM HEPES-OH, pH 7.9
200 mM KCl
5 mM $MgCl_2$
2.5 mM EDTA
0.5 mM EGTA
20% (v/v) Ficoll

TBE (5x)
175 mM Tris-HCl, pH 8.0
110 mM Boric acid
2.5 mM EDTA

2.2.11 Reverse transcriptase PCR (RT-PCR)

RT-PCR analysis of gene expression was as described (Meyer et al., 2002a). For human MTAP (Genbank # U22233) the primer pair tm110/tm111 (Tab. 2.2) was used. *Photinus pyralis* luciferase (Genbank # M15077) expression was determined with the primer pair B173/B174 (Tab. 2.2).

RNA-Isolation

Total RNA was isolated from 10 cm dishes grown to subconfluency using the Absolutely RNA® RT-PCR Miniprep Kit (Stratagene) according to the supplied instructions. The RNA concentration was determined by the optical density of the sample at 260 nm (OD_{260}) using a Ultrospec 2100pro photometer (Amersham) and a quartz cuvette (Hellma). An OD_{260} of 1 at a path length of 1 cm corresponded to a RNA concentration of 40 µg/ml. For the subsequent RT-PCR the RNA was diluted to 0.1 µg/µl in elution buffer. The integrity of the isolated RNA was checked on a 1% (w/v) agarose gel. If not used immediately, the RNA was quick frozen on dry ice and stored at -80°C.

RT-PCR reaction

Reverse transcription and amplification of the primer specific PCR-products were performed in the same reaction vial using the ProSTAR™ HF Single-Tube RT-PCR System (Stratagene) according to the provided manual. The reaction was performed in a total volume of 50 µl, containing 1.0 µl RNA (0.1 µg/µl), 5.0 µl 10x HF RT-PCR buffer, 1.0 µl forward primer (125 ng), 1.0 µl reverse primer (125 ng), and 1.0 µl dNTPs (10 mM each: dATP, dCTP, dGTP, dTTP). The reaction mix was first heated for 10 min at 65°C to denature the RNA, and then immediately cooled on ice. After the addition of 1.0 µl diluted Stratascript reverse transcriptase (2.5 U), cDNA synthesis was performed for 45 min at 42°C. Subsequently, the amplification of the specific PCR-products was started by the addition of 0.5 µl TaqPlus Precision DNA polymerase to each reaction. The PCR was performed in a gradient cycler (Mastercycler gradient, Eppendorf), using the following program: after a 2 min initial denaturation step at 95°C, 20-30 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 52-58°C and 1 min elongation at 68°C were performed. Five µl of the PCR reaction were run on a 1% (w/v) agarose gel. The PCR products were detected with UV light on a transilluminator and quantified using the program EasyWin32 (Herolab).

2.2.12 Reporter gene assay

Reporter gene assays with transiently transfected cells were done as described (Begitt et al., 2000). IFNα-responsive transcription was detected with a luciferase reporter gene (a kind gift of Dr. H. Hengel, RKI, Berlin) linked to five copies of an interferon-stimulated response element (ISRE, 5'-GTTTCACTTTCCCTA). An NF-κB-dependent luciferase reporter gene was kindly provided by Dr. C. Scheidereit (MDC, Berlin). Transfections for luciferase assays were performed in six independent experiments and the data presented are the means ± standard deviations. Cells grown in a 24-well plate were transfected with 0.15 µg of a luciferase reporter construct and 0.1 µg of β-galactosidase expression plasmid per well. Sixteen hours following transfection cells were stimulated for 6 h with IFNγ or IFNα. Subsequently, cells were rinsed with PBS and lysed in 150 µl glycylglycine lysis buffer per well. The lysates were centrifuged at 4°C, 16,000 x g and analysed for luciferase activity using the Luciferase Assay System (Promega) according to the supplied instructions. Emitted light was detected using a Microplate Luminometer LB 96V (EG&G Berthold, Bad Wildbad). In order to determine the β-galactosidase activity as an internal standard, 4 µl lysate were mixed with 56 µl of β-Gal reagent. The β-Gal reagent contained the colourless galactoside ONPG (o-nitrophenyl-β-D-galactopyranose, Sigma). ONPG hydrolysis by β-galactosidase, resulted in the release of galactose, and the yellow chromogenic compound, o-nitrophenol. The reaction was stopped after 5-20 min incubation at RT by the addition of 100 µl stop solution (0.5 M Na_2CO_3), and the reaction product o-nitrophenol was determined photometrically at an absorbance of 420 nm using a Tecan Safire Plate reader (Tecan, Crailsheim). The relative light units are given by the luciferase activity divided by the β-galactosidase activity. Calculation of standard deviations and graphical display were done using the program Prism (GraphPad Software; San Diego, CA).

Glycylglycine lysis buffer
25 mM Glycylglycine (Sigma)
15 mM $MgSO_4$
4 mM EGTA
1% (v/v) Triton X-100
1mM DTT
adjust pH with HCl to 7.8

Sodium phosphate buffer
100 mM NaH_2PO_4/Na_2HPO_4, pH 7.2

β-Gal reagent
211 µl Sodium phosphate buffer
66 µl ONPG (4 mg/ml, in sodium phosphate buffer)
3 µl 100x Mg solution

100x Mg solution
100 mM $MgCl_2$,
4.5 mM β-Mercaptoethanol

2.2.13 *In vivo* labelling and fluorography

Labelling reactions were performed with subconfluent monolayers of HeLa S3 cells essentially as described by Liu and Dreyfuss (1995). In brief, cells growing on 100 mm plates were washed twice with PBS and incubated in medium A [DMEM without L-methionine (Sigma), supplemented with 0.584 g/L L-glutamine (Gibco), 100 µg/ml streptomycin, 100 U/ml penicillin, and 5% fetal calf serum (dialysed against PBS overnight at 4°C, using a dialysis tube with a molecular weight cut-off of 14,000 Da, Roth). To inhibit protein synthesis, 100 µg/ml cycloheximide (Calbiochem) and 40 µg/ml chloramphenicol (Eurobio) were included. After 30 min the medium was replaced with fresh medium A containing 10 µCi/ml L-[*methyl*-^{3}H]methionine (Amersham). Labelling was performed for 3 h in the presence of IFNα and the protein synthesis inhibitors. Subsequently, cells were washed twice with ice cold PBS and lysed using 1 ml basic lysis buffer (BLB) supplemented with 0.5% (v/v) Triton X-100, 1 mM pervanadate, 0.1 mM DTT, 0.1 mM PMSF and Complete protease inhibitors (Roche). After 30 min incubation on ice, the lysates were centrifuged for 5 min at 16,000 x g at 4°C. The supernatants were used for immunoprecipitation of STAT1 using the following antibodies: either 2 µg E23 or 2 µg each of C24 and C136 (all Santa Cruz Biotechnology). The immunocomplexes were recovered with 40 µl (50% v/v slurry) protein A (Repligen; Waltham, MA) or protein A/G agarose (Santa Cruz Biotechnology), respectively, and washed four times with BLB containing 0.01% (v/v) Triton X-100 and 0.1 mM DTT followed by one wash in 0.01% (v/v) Triton X-100 in PBS. Bound proteins were eluted by boiling in 50 µl 2x SDS sample buffer (2.2.6). The immunoprecipitates were separated on precast reducing 4-12% NuPAGE gradient gels (Invitrogen) using MOPS running buffer. The gel was fixed in 10% acetic acid, 20% MeOH for 30 min, and was prepared for fluorography by washing with H_2O for 1 h, followed by soaking in 1 M sodium salicylate for 30 min essentially as described (Laskey and Mills, 1975). The dried gel was exposed for up to 2 weeks to photographic BioMaxMS films (Kodak, Stuttgart) at -80°C using a Biomax (Kodak) intensifier screen. Successful STAT1 immunoprecipitation was verified by Western blotting and silver staining. To check the efficacy of the protein synthesis inhibitors, one 10 cm plate HeLa S3 cells was incubated in medium A in the presence of 100 µg/ml cycloheximide and 40 µg/ml chloramphenicol, while another plate with an equal density of cells was grown in the absence of protein synthesis inhibitors. After 30 min incubation, 10 µCi/ml L-[^{35}S]methionine (Amersham) and IFNα were added to each plate and left on the cells for three hours. Cell lysis, STAT1 immunoprecipitation, and gel electrophoresis were as described. Gels were prepared for fluorography as before,

incorporated label was detected with the phosphoimaging system Storm 820 from Molecular Dynamics.

BLB (basic lysis buffer)	MOPS running buffer (20x)
10 mM Tris-HCl, pH 7.5	1 M Tris
150 mM NaCl	69.3 mM SDS
5 mM EDTA	20.5 mM EDTA

2.2.14 Protein expression and purification

GST-fusion proteins

Bacterial expression and purification of the GST-fusion proteins was performed as described (Begitt et al., 2000). In brief, the fusion proteins were expressed in *E. coli* (BL21 pLysS) and affinity-purified using glutathione-sepharose beads (Amersham Pharmacia) according to the manufacturer's instructions. The STAT1 N-domain (aa 1-129) was expressed either as a N-, or a C-terminal fusion protein to GST. A 1 L expression culture containing 100 µg/ml ampicillin was inocculated with 10 ml of a fresh overnight culture and grown at 37°C to an optical density of OD_{550} ~ 0.5. Subsequently, the culture was cooled down on ice to RT and protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Roth). After induction, protein expression was allowed to proceed for 16 h at 18°C. The culture was spun down for 15 min at 6000 x g (Avanti J-25 centrifuge; Beckman Coulter, Fullerton, CA) and resuspended in 100 ml GST lysis buffer per 1 L culture pellet. Protease inhibitors (Complete and 0.1 mM PMSF) as well as 1 mM DTT were included prior to cell lysis. Following three freeze/thaw cycles and three times ultrasonification (30 sec, cycle 5, 50%; Sonoplus UW 2070; Bandelin, Berlin), the lysate was centrifuged at 18,000 x g. One ml packed glutathione-sepharose beads were equilibrated in PBS, 0.1% (v/v) Triton X-100 and added to the cleared lysate, and binding was performed for 2 h at 4°C on a rotating wheel. Subsequently, the beads were washed four times with washing buffer including 1 mM DTT and protease inhibitors. Proteins were eluted in three steps with 300 µl of elution buffer, containing 15 mM glutathione (GSH, Sigma). Protein concentration was determined with the Bio-Rad Protein Assay (Biorad). Depending on the subsequent application, proteins were concentrated by ultrafiltration with a centriprep 50 device (Amicon, Bedford, MA) or dialysed against microinjection buffer (2.2.3).

GST lysis buffer
1x PBS
0.1% (v/v) Triton X-100
50 mM EDTA
2 mM EGTA
150 mM KCl

GST washing buffer
1x PBS
0.1% (v/v) Triton X-100
50 mM EDTA
2 mM EGTA

GSH elution buffer
50 mM Tris-HCl, pH 8.0
150 mM KCl
0.1% (v/v) Triton X-100
15 mM glutathione
5 mM DTT

PBS, pH 7.4 (HCl)
1.4 mM KH_2PO_4
4.3 mM Na_2HPO_4
2.7 mM KCl
137 mM NaCl

Strep-tagged proteins

Strep-tagged proteins were expressed and purified according to the instructions of the Strep tag manual (IBA). In brief, a 1 L *E.coli* (BL21 pLysS) expression culture was induced at an OD_{550} ~ 0.5 with 0.2 µg/ml anhydrotetracycline hydrochloride (Acros; Geel, Belgium). Protein expression was allowed for 5 h at 30°C. Following centrifugation for 15 min at 6000 x g the cell pellet was lysed in Strep lysis buffer (25 ml per 1 L culture) as described above. One ml streptactin beads (50% slurry, IBA) were equilibrated by three washes with lysis buffer and subsequently added to the lysate. Binding was performed for 2 h at 4°C on a rotating wheel. Following five washes with washing buffer (buffer W), elution was stepwise with 6 x 0.5 ml elution buffer (buffer E).

Strep lysis buffer
1x PBS
150 mM KCl
150 mM NaCl
2 mM EDTA
2 mM EGTA
0.1% (v/v) Triton X-100

Buffer W
100 mM Tris-HCl, pH 8.0
150 mM NaCl
1 mM EDTA
1 mM EGTA
0.1% (v/v) Triton X-100

Buffer E
2.5 mM Desthiobiotin (Sigma) in buffer W

2.2.15 Importin-α5 interaction assay

U3A cells grown in 10 cm dishes were transiently transfected with wild type STAT1 (pSTAT1-GFP) or the corresponding mutant expression plasmid lacking the N-domain (pSTAT1(ΔN)-GFP) as described above (2.2.2.2). Twenty-four hours after transfection, the cells were stimulated for 30 min with IFNγ, and subsequently were lysed in 0.5 ml import buffer complemented with 10 mM DTT, 1 mM vanadate, 0.05% (v/v) Triton X-100, and

protease inhibitors. Whole cell extracts were prepared as described in 2.2.5. Subsequently, STAT1 tyrosine phosphorylation was checked by Western blotting (2.2.7), and the lysates were normalised for equal concentrations of phosphorylated STAT1 using extracts from untransfected U3A, prepared as above. The normalised lysates were precleared for 1 h at 4°C using 30 µl streptactin beads (50% slurry, IBA), which were pre-equilibrated in import buffer for 30 min. For importin-α5 pull down assays 5 µg recombinant, Strep-tagged importin-α5 (kindly provided by Edda Schulz, AG Vinkemeier) was immobilised for 1 h at 4°C to 30 µl pre-equilibrated streptactin beads (50% slurry, IBA). The beads were washed three times to remove unbound importin-α5, and subsequently were mixed with the precleared cell lysates. Binding was performed for 2 h at 4°C on a rotation wheel. After four washes with 0.75 ml lysis buffer, the beads were resuspended in 30 µl of 2x SDS sample buffer. STAT1 bound to the immobilised importin-α5 was detected in a Western blot analysis using an α-phospho-STAT1(Y701) antibody (2.2.7).

Import buffer
20 mM HEPES-OH, pH 7.3
110 mM KAc
1 mM EDTA
2 mM MgAc

2.2.16 Mass spectrometric analysis

Analysis of STAT1 methylation

STAT1 was precipitated from U3A cells stably expressing Strep-tagged STAT1. Cells were left untreated or were treated with IFNα or -γ for 30 min. Tyrosine phosphorylation was confirmed by Western blotting. Cells (two to four 150 mm plates) were grown to 90% confluency and lysed by dounce homogenisation with 1 ml whole cell extraction buffer per plate (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM vanadate, 5 mM DTT, 0.1% (v/v) Triton X-100, 0.1 mM PMSF, protease inhibitors Complete, Roche). After 10 min centrifugation at 16000 x g the pooled extracts were combined with 40 µl (50% v/v slurry) of streptactin beads (IBA) and incubated at 4°C for 4 h or overnight. After washing in lysis buffer (four times) the beads were boiled in SDS-sample buffer and the eluted proteins were resolved on precast 4-12% NuPAGE gels (Invitrogen) using MOPS-SDS running buffer (2.2.13). Gels were stained with 0.1% (w/v) Coomassie-brilliant blue R250 (Merck) and destained in 40% MeOH, 10% acetic acid. The protein band representing 1-2.5 µg STAT1 (as determined by the intensity of Coomassie-blue staining) was excised, washed with 50% (v/v) acetonitrile in

25 mM ammonium bicarbonate, shrunk by dehydration in acetonitrile, and dried in a vacuum centrifuge. The gel pieces were re-swollen in 20 µl of 5 mM ammonium bicarbonate containing 600 ng AspN (sequencing grade, Roche). After 15 min, 10 µl of 5 mM ammonium bicarbonate was added to keep the gel pieces wet during enzymatic cleavage (37°C, over night). To extract the peptides, 30 µl of 0.5% (v/v) trifluoroacetic acid (TFA) in acetonitrile was added, the samples were sonicated for 5 min, and the separated liquid was taken to dryness under vacuum before the samples were dissolved in 10 µl of 0.1% (v/v) TFA, 5% (v/v) acetonitrile in water. The peptides were purified using a C18 reversed-phase minicolumn fitted into a micropipette tip (ZipTip C18, Millipore), prior to mass spectrometric analysis. The purification was carried out according to the manufacturer's manual. Peptides were eluted from the ZipTips with 2.5 µl of 60% (v/v) acetonitrile, 0.3% (v/v) TFA in water. For nanoscale liquid chromatography/electrospray ionisation mass spectrometry (nanoLC-ESI-MS), the samples were reconstituted in 5 µl of 0.1% (v/v) TFA, 6% (v/v) acetonitrile in water.

Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) measurements were performed on a Voyager-DE STR BioSpectrometry Workstation MALDI-TOF mass spectrometer (Perseptive Biosystems; Framingham, MA). One µl of the peptide solution was mixed with 1 µl of alpha-cyano-4-hydroxycinnamic acid matrix solution consisting of 10 mg of matrix dissolved in 1 ml of 0.3% TFA in acetonitrile-water (1:1, v/v). From the resulting mixture 1 µl was applied to the sample plate. Samples were air-dried at ambient temperature. Measurements were performed in the reflection mode at an acceleration voltage of 20 kV, 70% grid voltage and a delay of 200 ns. Each spectrum obtained was the mean of 256 laser shots. The program FindMod (available on the internet at expasy.ch/tools/findmod) was used to interpret the MS spectra of protein digests. Peptides from AspN-digested STAT1 covered 59% of the protein (SwissProt accession No. P42224).

Tandem MS analyses were performed on a quadrupole orthogonal acceleration time-of-flight mass spectrometer Q-Tof Ultima (Micromass) equipped with a Z-spray nanoelectrospray source. A Micromass CapLC liquid chromatography system was used to deliver the peptide solution to the electrospray source. Five µl of the sample was injected using 0.2% formic acid in water at a flow rate of 20 µl/min (eluent C) and concentrated on a precolumn (PepMap C18, 5 µm, 100 Å, 5 mm x 300 µm i.d., LC Packings, Dionex; Sunnyvale, CA). Peptides were eluted onto an analytical column (PepMap C18, 3 µm, 100 Å, 150 mm x 75 µm i.d., LC Packings) and separations were performed at an eluent flow rate of 200 nl/min which was achieved by splitting the flow (5 µl/min) of pumps A and B. Mobile

phase A was 0.1% formic acid (v/v) in acetonitrile-water (5:95, v/v) and B was 0.1% formic acid in acetonitrile-water (8:2, v/v). Runs were performed using a concave gradient of 3-65% B in 60 min. The mass spectrometer was operated in the positive ion mode using PicoTip spray capillaries (New Objective; Woburn, MA). To perform MS/MS experiments data-dependent acquisition (DDA) was employed. The collision gas was argon at a pressure of 6.0×10^{-8} bar in the collision cell and the collision energy was between 25 and 55 eV depending on the charge state and the mass of the precursor ion. The processed MS/MS spectra (MassLynx version 4.0 software) were compared with the theoretical b- and y-ions of AspN-fragments of the protein.

Detection of ratjadonylated CRM1 peptides

Peptide labelling was performed essentially as described by Kudo et al. (1999). One µg of a peptide of human CRM1 (residues 513-530; Biosynthan, Berlin), containing the conserved Cys^{528}, was treated with or without 10 µg ratjadone in 15 µl labelling buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl). Following overnight incubation at 37°C the samples were analysed by mass spectrometry. Samples were diluted with 5 µl of 1% (v/v) formic acid in water and purified over a C18 reversed-phase microcolumn, ZipTip (Millipore), according to the manufacturer's manual. Peptides were eluted from the ZipTips with 5 µl of 70% (v/v) acetonitrile, 0.25% (v/v) formic acid in water. Mass spectrometric analyses of unmodified and modified CRM1 peptides were performed as previously described (Kleuss and Krause, 2003). Briefly, MALDI-MS measurements were performed on a Voyager-DE STR BioSpectrometry Workstation MALDI-TOF mass spectrometer (Perseptive Biosystems, Inc.) using an alpha-cyano-4-hydroxycinnamic acid matrix. In order to verify the sequences of CRM1(513-530) and the position of modification, MS/MS analyses were performed by nanoelectrospray quadrupole TOF (Q-Tof Ultima; Manchester, UK) and MALDI TOF-TOF (4700 Proteomics Discovery System; Applied Biosystems) mass spectrometry.