3 Materials and methods

Materials used to carry out the experiments are listed along with the descriptions of the specific methods that they were used for, except for common chemicals that were necessary for most experiments. These are therefore listed here.

Standard laboratory chemicals Inorganic salts (NaCl, KCl, etc.), bovine serum albumin (BSA), β-mercaptoethanol (β-ME), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), glucose, imidazole, sodium dodecyl sulfate (SDS), Triton X-100 (all from Sigma, Schnelldorf), peppermint tea (Herba, Aurich), boric acid, ethanol, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES), hydrochloric acid (HCl), methanol, sodium hydroxide (NaOH; Merck, Darmstadt), agar-agar, tryptone/peptone, urea, yeast extract (Roth, Karlsruhe), agarose (low EEO), ethidium bromide solution, glycerol, Tris(hydroxymethyl-)aminomethan (Tris), Tween-20 (Applichem, Darmstadt).

3.1 Culture and transfection of mammalian cells

All mammalian cell lines were grown at 37°C and 5% CO_2 or 7% CO_2 (DMEM-based media) in an humidified atmosphere. Cells were subcultured every 3–4 days by detaching them with trypsin/EDTA and seeding in new flasks at a dilution of 1:5–1:10. For transfection, cells were seeded in 35-mm dishes without glass coverslips for Western blot experiments or with glass coverslips for imaging experiments at a density of $1–2\times10^5$ cells per dish. For some experiments, glass coverslips were coated with poly-l-lysine to enhance adherence of cells. Cells were seeded in 60-mm dishes for immunoprecipitation (IP) experiments at a density of $0.5–1\times10^5$ cells per dish.

- **MEM medium for HEK293 cells** MEM medium with Earle's salts supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin.
- **DMEM medium for HEK293 cells** DMEM medium with 4.5 g/l glucose supplemented with 10% FBS, 4 mM glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin.
- **Medium for COS-7 cells** DMEM medium with 4.5 g/l glucose supplemented with 10% FBS, 4 mM glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin.

- **Medium for RBL-2H3 cells** MEM medium with Earle's salts supplemented with 18% FBS, 2 mM glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin.

Transfection of COS-7 and HEK293 cells was performed 1–2 days after seeding at a confluency of 50–70% using the Fugene6 transfection reagent according to the manufacturer's protocol. The amount of plasmid DNA and Fugene6 used for transfection was 2 µg and 4 µl per 35-mm dish and 4 µg and 8 µl per 60-mm dish, respectively. In cases where less cDNA-containing plasmid was necessary, empty pcDNA3 expression vector was used to keep the total amount of plasmid constant. Experiments were performed 1–2 days after transfection.

Because the transfection efficiency of RBL-2H3 cells achieved by lipofection methods was insufficient, the nucleofection technique was used for gene silencing studies in RBL-2H3 cells. It is a modified version of electroporation that follows the same principles of voltage-driven DNA transfer, but uses proprietary cell type-specific solutions and current protocols. Per nucleofection, 6×10^6 cells and 5 µg DNA were used. RBL-2H3 cells detached by trypsin were counted, and the appropriate number of cells was pelleted for 10 min at $200\times$g. Cells were resuspended in Nucleofector solution V containing the plasmid DNA. Nucleofection was performed with protocol T-20 of the Amaxa nucleofector. The nucleofected cells were diluted in medium, and the total amount of cells was plated in either 6 35-mm dishes or all wells of a 24-well plate. On the next day, the medium was replaced by fresh medium.

Materials for cell culture and transfection HEK293 cells (Institut für Pharmakologie, Berlin, and ATCC, Manassas, USA), COS-7 cells, RBL-2H3 cells (ATCC, Manassas, USA), MEM dry medium, DMEM dry medium (PAN, Aidenbach), penicillin/streptomycin, glutamine, non-essential aa, trypsin/EDTA (PAA, Pasching, Austria), foetal bovine serum (Gibco *via* Invitrogen, Karlsruhe), glass coverslips (Menzel, Braunschweig), Fugene6 transfection reagent (Roche Applied Science, Mannheim), Cell line Nucleofector Kit V (Amaxa, Köln).

3.2 Molecular biology

All techniques used in this section are based on standard methodology of molecular biology, which is extensively described in Sambrook and Russel (2001). Therefore, they will not be explained in detail here. The following sections on the generation of expression plasmids, on multiplex polymerase chain reaction (PCR) applications, and Northern blotting will rather focus on the specific strategies employed, allowing them to be reproduced.

3.2.1 Generation of expression plasmids

Expression plasmids used in this study were based on the pcDNA3.1-V5/His/TOPO and pcDNA3 vectors. In addition to elements necessary for propagation and selection in *E. coli*, they contain a CMV promoter and a polyadenylation signal driving expression of genes of interest in mammalian cells. PCR products containing single 3'-deoxyadenosine overhangs can be directly subcloned into the linearized pcDNA3.1-V5/His/TOPO vector by virtue of a topoisomerase fused to its single 3'-deoxythymidine overhangs. Modified versions of the pcDNA3 expression vector were used to generate either 5' or 3' fusions of cDNA with sequences encoding epitope tags or fluorescent proteins. These vectors were constructed in the group and are listed in Table 3.1.

Generally, forward primers used for PCR cloning contained an optimized ribosomal binding site (Kozak consensus sequence GCCACC; Kozak, 1987) immediately upstream of the ATG start codon. Additionally, appropriate restriction sites or stop codons were often incorporated by the reverse primers. All constructs generated by PCR were verified by sequencing either on a ABI377 sequencer (ABI *via* Perkin Elmer, Rodgau) or through a commercial sequencing service (SeqLab, Göttingen).

Table 3.1: Vectors for expression and epitope tagging

Vector	Source
pcDNA3	Invitrogen, Karlsruhe
pcDNA3.1-V5/His/TOPO	Invitrogen, Karlsruhe
pcDNA3-CCFP	M. Schaefer, Berlin
pcDNA3-CYFP	M. Schaefer, Berlin
pcDNA3-NCFP	P. Voigt, Berlin
pcDNA3-NYFP	P. Voigt, Berlin
pcDNA3-FLAG	N. Hellwig, Berlin
pFastBac1	Invitrogen, Karlsruhe

Materials for expression plasmid generation QIAprep spin Miniprep Kit (Qiagen, Hilden), Nucleobond AX Maxi Kit (Macherey-Nagel, Düren), Expand High Fidelity PCR system (Roche Applied Science, Mannheim), Bac-to-Bac Baculovirus Expression System, pcDNA3.1/V5-His TOPO TA Expression kit, Superscript II/III reverse transcriptase, Trizol, 1 kb DNA ladder, TOP10 chemically competent *E. coli* (Invitrogen, Karlsruhe), restriction endonucleases (New England Biolabs, Frankfurt am Main), LigaFast rapid DNA ligation system (Promega, Mann-

heim), DYEnamic ET Cycle Sequencing Kit (Amersham Pharmacia, Freiburg), oligonucleotides (Roth, Karlsruhe, and BioTeZ, Berlin).

p101 deletion mutants

Deletion mutants of p101 were generated by PCR. Porcine p101 (in pcDNA3, see Table 3.3) was used as the template. After subcloning of PCR products into pcDNA3.1-V5/His/TOPO, constructs were either directly used for expression studies or transferred into pcDNA3-based vectors for N- or C-terminal fusion of the deletion mutants to cyan fluorescent protein (CFP) or – for some constructs – yellow fluorescent protein (YFP). For fusion with a 3′ tag sequence, the reverse primers contained an Xba I restriction site to ensure the correct transition between reading frames. The restriction site was located upstream of the artificial stop codon that terminates translation of the deletion mutant if it is not removed by subcloning. The PCR primers used for each deletion mutant are given in Table 3.2 along with the position of the CFP tag. The majority of the deletion mutants was subcloned into pcDNA3-NCFP *via* Hind III and Not I for N-terminal fusion or into pcDNA3-CCFP *via* Hind III and Xba I for C-terminal fusion with CFP. Unwanted mutations introduced by the PCR reactions led to deviations from the general cloning scheme for some constructs. For reasons of clarity, these additional subcloning steps are not described here. Some deletion mutants were already constructed as part of the preceding diploma thesis (Voigt, 2003). For the sake of completeness, they are included in Table 3.2 as well.

Table 3.2: Primers and tag positions of p101 deletion mutants

aa	forward primer	reverse primer	tag pos.
1–100	5′-GCC ACC ATG CAG CCA GGG GCC-3′	5′- TTA ATC TGA GTC TGG CGG GA –3′	NT
1–131	″	5′- TTA ATC TAG AAA GGT GAG CAG TTC CTG -3′	NT
1–150	″	5′- TTA GCC CTG CTC CGC CCT –3′	NT
1–175	″	5′- TTA GAA CTC AGC CTG CAC CT –3′	NT
1–200	″	5′- TTA GGC ATG CAG GAG CAG G –3′	NT
1–223	″	5′- TTA ATC TAG AGT CTT GGA CTG CAA CCT -3′	NT
1–250	″	5′- TTA CCG GGC CTC AGC TGC –3′	NT
1–275	″	5′- TTA AGG TTT GGC GGT GTC TAA –3′	NT
1–300	″	5′- TTA GAT GTC GAA GCT GTC CT –3′	NT
1–333	″	5′- TTA ATC TAG ATC CTC TTC CTC GTC CT -3′	NT
1–613	″	5′- TTA ATC TAG AAT GCC GAG GTA GTG GG -3′	NT
25–333	5′- GCC ACC ATG AGC CTC AGC CGC CG –3′	5′- TTA ATC TAG ATC CTC TTC CTC GTC CT -3′	CT
50–333	5′- GCC ACC ATG AGG GAT CCG GGC CAC –3′	″	CT

continued on next page

aa	forward primer	reverse primer	tag pos.
75–333	5′- GCC ACC ATG TAT GAC CTC CTC GCG C –3′	″	CT
249–613	5′- GCC ACC ATG GAG GCC CGG CAG TG -3′	5′- TTA ATC TAG AAT GCC GAG GTA GTG GG -3′	NT/CT
249–877	″	5′- TTA ATC TAG AGG CAG AGC TCC GCT G -3′	CT
517–771	5′- GCC ACC ATG GTC GTG GTC TTC GGC T -3′	5′- TTA ATC TAG AGC CTC TGT GCT GGA GT –3′	NT
517–877	″	5′- TTA ATC TAG AGG CAG AGC TCC GCT G -3′	NT
630–800	5′- GCC ACC ATG GTC CTG TGC CAG TCC C -3′	5′- TTA GTC CAC TTT GAT CTG CGA –3′	NT
630–825	″	5′- TTA CTG CAG GAT CTT CCT CTC –3′	NT
630–850	″	5′- TTA GGG CCT CTG GGG TGG –3′	NT
630–877	″	5′- TTA ATC TAG AGG CAG AGC TCC GCT G -3′	CT
650–842	5′- GCC ACC ATG CCC ATC CTG GCG GAC A –3′	5′- TTA GCT CTT CTC AGG CTT GTA G –3′	NT
650–850	″	5′- TTA GGG CCT CTG GGG TGG –3′	NT
650–877	″	5′- TTA ATC TAG AGG CAG AGC TCC GCT G -3′	CT
675–877	5′- GCC ACC ATG GAG CTG ACC TTC ATC AC –3′	″	CT
700–877	5′- GCC ACC ATG ACA CGT GCC ATC AAG GC –3′	″	CT
725–877	5´- GCC ACC ATG ACA CTA CAG ATA ATT TAC AG –3´	″	CT

aa: amino acids, NT: CFP at N terminus of deletion mutant, CT: CFP at C terminus of deletion mutant.

Cloning of p87^{PIKAP}

For the cloning of p87^{PIKAP}, an RT (reverse transcription)-PCR-based strategy was used. First, total RNA from murine CD11c-positive DCs was reverse transcribed with Superscript II reverse transcriptase and an oligo(dT) primer, converting polyadenylated mRNA to single-stranded cDNA. In a second step, p87^{PIKAP} cDNA was amplified with specific primers that were designed based on the GenBank mRNA entry BC028998, which was identified to encode a putative p101 homologue (see section 4.1.6). The 5′ end of the forward primer (5′-CCT CCC CCA TAC AGG ACA GA-3′) was located 24 bp upstream of the putative start codon and that of the reverse primer (5′-GTG GGG CTG TCA GTG TAA ATG-3′) 99 bp downstream of the stop codon. The PCR product was subcloned into pcDNA3.1-V5/His/TOPO. Five independent clones were sequenced. Except for two point mutations and alternative splicing at one intron-exon boundary, they were identical to the coding sequence deposited in BC028998 (see section 4.2.1). To obtain both splice variants without mutations, clones were combined using restriction sites within the cDNA. The sequences of both variants were submitted to the DDBJ/EMBL/GenBank data bases under the accession numbers AY753194 and DQ295832.

Both splice variants of p87^{PIKAP} were then subcloned into pcDNA3-NCFP and pcDNA3-NYFP *via* Acc65 I and Xho I. To allow C-terminal fusion of p87^{PIKAP} to CFP, YFP, and FLAG tags, a 3′ Xba I restriction site was introduced by PCR with the forward primer 5′-GCC ACC ATG GAG AGC TCA GAT GTG-3′, which also contained an optimized ribosomal binding site

(underlined), and the reverse primer 5′-TCT AGA TGG ATG ATG CCA GAG AAT GTG-3′ (Xba I site underlined). The PCR products were first subcloned into pcDNA3.1-V5/His/TOPO and then into pcDNA3-CCFP, pcDNA3-CYFP, and pcDNA3-FLAG *via* Hind III and Xba I.

To obtain short hairpin RNA (shRNA) vectors for the use in cell lines derived from rats (see section 3.2.2), it was necessary to first clone the rat orthologue of p87^{PIKAP} to obtain its sequence. A corresponding mRNA sequence was deposited in the ENSEMBL data base by automated annotation routines (ENSEMBL gene ENSRNOG00000003992). Based on that sequence, primers were selected to amplify the rat p87^{PIKAP} cDNA (forward: 5′-TCC TTC CCT TAC AGG ACA GGC-3′, reverse: 5′-GAC GCC CAG TGA GGC TGT C-3′).

The heart of a 2 day-old Wistar rat was homogenized in 1 ml Trizol reagent to prepare total RNA. The extracted and washed RNA was resuspended in 100 µl RNAse-free water. cDNA was generated from 1.5 µg total RNA by RT-PCR with Superscript III and oligo(dT) primer. A PCR product of the expected size was obtained. It was subcloned into pcDNA3.1-V5/His/TOPO. Sequencing of several clones consistently yielded two point mutations with respect to the annotated sequence. Because these mutations render the sequence identical to the wild-type murine orthologue on both cDNA and protein level, it was concluded that the clones obtained have the correct sequence. The coding sequence was deposited in the DDBJ/EMBL/GenBank data bases under accession number EF207569.

The cDNA was further subcloned into pcDNA3-NCFP and -NYFP for N-terminal fusion with CFP and YFP *via* Acc65 I and Xho I. For C-terminal fusion with CFP, YFP, and FLAG, another round of PCR was performed with the same primers as for the murine variant of p87^{PIKAP} (see above). After subcloning of the PCR product into pcDNA3.1-V5/His/TOPO and sequencing, the cDNA was transferred to pcDNA3-CCFP, -CYFP, and -FLAG *via* Acc65 I and Xba I.

Vectors for baculovirus generation

To allow purification of p110γ and p87^{PIKAP} protein, vectors based on pFastBac1 were generated for expression in insect cells. A His tag-containing version of pFastBac1 was generated based on a C-terminal fragment of p101 (aa 650–841). This construct was generated by two rounds of PCR. First, a part of the cDNA of porcine p101 was amplified with the forward primer 5′-GGT ACC ATC GAG GGA AGG ATT CCC ATC CTG GCG GAC ATG-3′, introducing an Acc65 I restriction site (underlined) followed by four codons encoding a factor Xa protease recognition site (aa sequence IEGR), and the reverse primer 5′-AAG CTT AGC TGC TCT TCT CAG GCT TGT AG-3′ that contains a Hind III restriction site. After gel purification of the product, a second round of PCR was performed with the same reverse primer but a different forward primer that adds a His tag-encoding sequence preceded by an ATG codon to the product of the first PCR (5′-CCA TGG GAC ATC ACC ATC ACC ATC ACG GTA CCA TCG AGG GAA GG-3′). The resulting 633 bp fragment was subcloned into pcDNA3.1-V5/His/TOPO

and transferred to pFastBac1 *via* Sac I and Hind III.

In this His tag-containing version of pFastBac1, the C-terminal part of p101 was then replaced by the full-length sequence of $p87^{PIKAP}$. To this end, murine $p87^{PIKAP}$ cDNA was amplified with a forward primer that likewise contained a Acc65 I restriction site and the factor Xa recognition site (5′-GGT ACC ATC GAG GGA AGG ATT ATG GAG AGC TCA GAT GTG-3′) and the same reverse primer as for the initial RT-PCR-based cloning of $p87^{PIKAP}$ (5′-GTG GGG CTG TCA GTG TAA ATG-3′). The amplified cDNA was cloned into pcDNA3.1-V5/His/TOPO and transferred to the His tag-containing pFastBac1 *via* Acc65 I and Hind III.

Using the same approach, an expression vector for His-tagged p110γ was generated. The p110γ cDNA was amplified with the forward primer 5′-GGT ACC ATC GAG GGA AGG ATT ATG GAG CTG GAG AAC TAT-3′ and the reverse primer 5′-CGT CTC AAG CTT AGG CTG AAT GTT TCT CTC-3′ that introduces a BsmB I restriction site (underlined). After subcloning of the resulting PCR product into pcDNA3.1-V5/His/TOPO, the cDNA was excised with Acc65 I and BsmB I and used to replace the p101-derived fragment in the His tag-containing version of pFastBac1, which was digested with Acc65 I and Hind III. BsmB I was used to generate a Hind III-compatible overhang, because Hind III recognition sites are present within the p110γ cDNA.

Similarly, a construct was prepared that encodes a His-tagged p110γ with a tobacco etch virus (TEV) protease instead of a factor Xa recognition site for removal of the His tag. The p110γ cDNA was amplified by PCR with the forward primer 5′-GGT ACC GAA AAC CTG TAT TTT CAG GGC GCT ATG GAG CTG GAG ACC TAT A-3′, which contains an Acc65 I restriction site and encodes a TEV protease recognition site (aa sequence ENLYFQG). The reverse primer was the same one as above (5′-CGT CTC AAG CTT AGG CTG AAT GTT TCT CTC-3′). After subcloning into pcDNA3.1-V5/His/TOPO, the 5′ part of the PCR product encoding the TEV protease recognition site and parts of p110γ was excised with Acc65 I and BstE II and used to replace the respective parts in the above described expression vector for His-tagged p110γ.

An expression vector for wild-type untagged murine $p87^{PIKAP}$ was obtained by subcloning the coding sequence of its shorter splice variant from pcDNA3.1-V5/His/TOPO to pFastBac1 *via* Acc65 I and Hind III.

To obtain bacmid DNA necessary for the transfection of Sf21 cells (see section 3.5.1), the pFastBac1-based constructs were transformed into DH10Bac *E. coli*. After incubation at 37°C for 6 h during which transposon-mediated recombination of the expression cassette from pFastBac1 to the precursor bacmid bMON14272 (based on a bacmid generated by Luckow *et al.*, 1993) takes place, cells were plated on selective plates, resulting in growth of white colonies for recombinant bacmid. After confirming the correctness of picked colonies by replating, bacmid DNA was prepared by standard mini prep procedures. To verify proper insertion of the expression cassette, PCRs were performed with specific sets of primers hydridizing to the bacmid and the cDNA that has been inserted, so that a product was only obtained for bacmids containing

cDNA transposed from pFastBac1.

For Gβ_1 and Gγ_2-His, baculovirus stocks were used that have been generated previously (Leopoldt *et al.*, 1998).

Miscellaneous constructs

p110γ-FLAG The p110γ-encoding cDNA was excised from p110γ-YFP *via* Acc65 I and Xba I and subcloned into pcDNA3-FLAG.

βARK-CT The G$\beta\gamma$-binding C terminus of the β-adrenergic receptor kinase 1 (βARK-CT) was amplified by PCR from βARK1-DsRed (from R. Schulz, München). The forward primer 5′-GCC ACC ATG GGC ACC AAA AAC AAG CAG TTG G-3′ introduced a Kozak sequence, an ATG start codon, and an additional Gly-encoding triplet upstream of the βARK-CT-encoding cDNA sequence. The reverse primer 5′-TTA ATC TAG ACC GTT GGC ACT GCC G-3′ contains a Xba I restriction site (underlined) downstream of the TTA antisense stop codon. The amplified βARK-CT construct spans aa 542–689 of βARK1. It was subcloned first into pcDNA3.1-V5/His/TOPO and then transferred to pcDNA3-CCFP *via* Hind III and Xba I and to pcDNA3-NCFP *via* Hind III and Not I.

PDE3B The plasmid encoding murine PDE3B-FLAG was a gift from Eva Degerman (Lund, Sweden). It was used to generate fluorescent protein-tagged versions of PDE3B. PCR was performed with the forward primer 5′-GCC ACC ATG AGG AAA GAC GAG CGC-3′ (Kozak sequence underlined) and the reverse primer 5′-TTA GCT CGA GTC AAA CAT TTG TTC TTC CTC TTC A-3′ (introduced Xho I site underlined). After subcloning into pcDNA3.1-V5/His/TOPO, 3′ fusions with CFP-, YFP-, or FLAG-encoding sequences were generated *via* Hind III and Xho I. For 5′ fusions, the CFP- or YFP-encoding sequence was transferred to PDE3B-containing pcDNA3.1-V5/His/TOPO *via* Nhe I and Hind III.

Previously generated plasmids Plasmids that were used but not generated in this thesis are listed in Table 3.3. Details concerning their generation can be found in the references given.

Table 3.3: Further expression vectors

Construct	Vector backbone	Source	Reference
CFP	pECFP-C1	Invitrogen, Karlsruhe	–
YFP	pEYFP-C1	Invitrogen, Karlsruhe	–
CFP-YFP tandem	pECFP-C1	N. Hellwig, Berlin	Hellwig *et al.* (2004)
TRPV1-FLAG	pcDNA3	N. Hellwig, Berlin	Hellwig *et al.* (2005)
CFP-p101	pcDNA3-NCFP	P. Voigt, Berlin	diploma thesis
p101	pcDNA3	C. Brock, Berlin	Brock *et al.* (2003)
CFP-p101	pECFP-C1	C. Brock, Berlin	″
p101-CFP	pcDNA3-CCFP	C. Brock, Berlin	″
YFP-p101	pEYFP-C1	C. Brock, Berlin	″
p101-YFP	pcDNA3-CYFP	C. Brock, Berlin	″
p110γ	pcDNA3	C. Brock, Berlin	″
YFP-p110γ	pEYFP-C1	M. Michalke, Berlin	″
p110γ-YFP	pcDNA3-CYFP	C. Brock, Berlin	″
CFP-p110γ	pECFP-C1	M. Michalke, Berlin	″
p110γ-CFP	pcDNA3-CCFP	C. Brock, Berlin	″
CFP-p110γ(K833R)	pECFP-C1	C. Brock, Berlin	″
YFP-p110β	pEYFP-C1	M. Michalke, Berlin	″
CFP-p110β	pEYFP-C1	M. Michalke, Berlin	″
Gβ_1	pcDNA3	M. Michalke, Berlin	″
Gγ_2	pcDNA3	M. Michalke, Berlin	″
fMLP receptor	pcDNA3	C. Brock, Berlin	″
p85α	pcDNA3	C. Brock, Berlin	Brock (2002)
YFP-Grp1-PH	pEYFP-C1	C. Brock, Berlin	″

3.2.2 Generation of plasmids for RNAi

RNA interference (RNAi) is an evolutionarily conserved response against exogenous viral pathogens and endogenous parasitic nucleic acids such as transposons, but it also functions in the regulation of protein-coding genes (see Hannon, 2002, for review). It was first discovered in plants (then called cosuppression; see Napoli *et al.*, 1990; Sen & Blau, 2006), but later also found to be active in *C. elegans* and to depend on double-stranded RNA (Fire *et al.*, 1998). The identification of 21-bp double-stranded RNA molecules as stable intermediates of this process initiated the use of short interfering RNA (siRNA; see Hamilton & Baulcombe, 1999; Elbashir *et al.*, 2001a). The system employed here uses plasmid-encoded short hairpin RNA (shRNA) rather than synthetic 21-bp siRNA molecules (Brummelkamp *et al.*, 2002). RNA hairpins consisting of the 21-bp sense target sequence followed by a short loop and the antisense target sequence are transcribed by RNA polymerase III under the control of an H1 RNA promoter.

Target sequences in $p87^{PIKAP}$ cDNA were selected so that both murine and rat versions of $p87^{PIKAP}$ can be silenced by the shRNA constructs. To select optimal sequences, the RNAi designer online tool (Invitrogen, http://www.invitrogen.com/rnai) was used, which is partly based on Tuschl's rules for siRNA generation (Elbashir *et al.*, 2001b). The accordingly designed primers are listed in Table 3.4. They are designated by their start position within the murine $p87^{PIKAP}$ coding sequence. The vectors were constructed following the manufacturer's recommendations. Briefly, top and bottom strand oligonucleotides were annealed and then ligated into linearized pENTR/H1/TO vector. The supplied lacZ2.1 control oligonucleotide duplex was used as a control. Clones with the correct inserts were then used for gene knockdown experiments.

Table 3.4: Primers for shRNA vector generation

Primer	Sequence
shRNA p87 104 top	5′-CAC CGG AGA TGG TCT CTG CAC AAG ACG AAT CTT GTG CAG AGA CCA TCT CC-3′
shRNA p87 104 bottom	5′-AAA AGG AGA TGG TCT CTG CAC AAG ATT CGT CTT GTG CAG AGA CCA TCT CC-3′
shRNA p87 958 top	5′-CAC CGC CTT GGA TCT ACA AGG ATT TGA GAA AAT CCT TGT AGA TCC AAG GC-3′
shRNA p87 958 bottom	5′-AAA AGC CTT GGA TCT ACA AGG ATT TTC TCA AAT CCT TGT AGA TCC AAG GC-3′
shRNA p87 1002 top	5′-CAC CGT GTC CAC CGA CAG TGG AAT TGA GAA ATT CCA CTG TCG GTG GAC AC-3′
shRNA p87 1002 bottom	5′-AAA AGT GTC CAC CGA CAG TGG AAT TTC TCA ATT CCA CTG TCG GTG GAC AC-3′
shRNA p87 1469 top	5′-CAC CGG TAT GAA AGC ACT GTC AAC ACG AAT GTT GAC AGT GCT TTC ATA CC-3′
shRNA p87 1469 bottom	5′-AAA AGG TAT GAA AGC ACT GTC AAC ATT CGT GTT GAC AGT GCT TTC ATA CC-3′

Materials for shRNA vector generation BLOCK-iT Inducible H1 RNAi Entry Vector Kit (Invitrogen, Karlsruhe). For further materials see section 3.2.1.

3.2.3 Multiplex and semi-quantitative RT-PCR

To assess mRNA expression of all PI3Kγ subunits in parallel, a multiplex PCR assay was established. Primers were selected for p110γ, p101, p87$^{\text{PIKAP}}$, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) so that fragments of similar but distinguishable size were amplified. Primer sequences are given in Table 3.6. All of the generated fragments spanned at least one intervening intron on the genomic level so that contaminations from genomic DNA would result in considerably longer PCR products. Moreover, each fragment contained a unique restriction site, allowing routine confirmation of the fragment identity by simple restriction digests. The characteristics of the PCR products are summarized in Table 3.5.

Table 3.5: Properties of multiplex PCR fragments

Fragment	Species	Target mRNA	Span (bp)	Size (bp)	Restriction site
GAPDH	mouse	NM_001001303	521–1061	541	Apa I
p110γ	mouse	NM_020272	3245–3648	404	Bcl I
p101	mouse	NM_177320	2263–2611	349	Xba I
p87$^{\text{PIKAP}}$	mouse	BC028998	2313–2598	286	Hind III
GAPDH	rat	AF106860	1312–1852	541	Apa I
p110γ	rat	XM_234053	3069–3472	404	Bcl I
p101	rat	ENSRNOG00000023428	2265–2616	352	Xba I
p87$^{\text{PIKAP}}$	rat	ENSRNOT00000005405	2082–2373	291	BamH I

Total RNA of rat heart and RBL-2H3 cells was prepared with Trizol reagent. RNA of murine heart, spleen, splenocytes, and different leukocyte subtypes was a gift from M. Dorner (Robert Koch-Institut, Berlin). Briefly, these samples were prepared by the following procedures (for a more detailed description see Dorner *et al.*, 2002; Löhning *et al.*, 2003). Macrophages were obtained from bone marrow of the femora of C57BL/6 mice. Splenocytes were derived from spleens of C57BL/6 mice. Specific antibodies and magnetic beads were used for magnetic cell sorting of leukocyte subpopulations from splenocytes. The purity of the cell populations was verified by flow cytometry using specific fluorescence-labeled antibodies. From these cells, either total or poly(A^+) RNA was prepared.

Total RNA (0.5–1.5 µg) or mRNA (5–50 ng) was reverse-transcribed with Superscript III and oligo(dT) primers. Multiplex PCR was performed with *Taq* polymerase in the supplied magnesium-free buffer supplemented with 1.5 mM $MgCl_2$. The PCR protocol was as follows:

initial denaturation at 94°C for 2 min, 24 amplification cycles (denaturation at 94°C for 20 s, annealing at 60°C for 45 s, extension at 72°C for 45 s), final extension at 72°C for 5 min. The primers are listed in Table 3.6. Initially, identities of the fragments obtained were confirmed by sequencing and routinely checked by restriction digests later on. Aliquots of the reactions were run on 1.5% agarose gels and documented with a CCD-based camera system (LAS-1000, Fujifilm *via* Raytest, Straubenhardt).

Table 3.6: Primers for multiplex PCRs

Primer	Sequence	Species
GAPDH forward	5′-TTA GCC CCC CTG GCC AAG G-3′	mouse, rat
GAPDH reverse	5′-CTT ACT CCT TGG AGG CCA TG-3′	mouse, rat
p110γ forward	5′-TCC TGG GCA TCA ATA AAG AGA G-3′	mouse, rat
p110γ reverse	5′-GGG CCC TAG CAC CAC ATA TC-3′	mouse
p101 forward	5′-AAG CCG GAG GAG CTA GAC TC-3′	mouse
p101 reverse	5′-GCA GAG CCC CAC TGA ATG TC-3′	mouse
$p87^{PIKAP}$ forward	5′-GGA CGG ACG GCG GAC TTT C-3′	mouse
$p87^{PIKAP}$ reverse	5′-GTG GGG CTG TCA GTG TAA ATG-3′	mouse
p110γ reverse	5′-GGG CTC TAG CAC CAA ATA TCA A-3′	rat
p101 forward	5′-GAA GCC GGA GGA ACT AGA TTC-3′	rat
p101 reverse	5′-GGG CAG AGC ACC ACT GAA AG-3′	rat
$p87^{PIKAP}$ forward	5′-GGA TGG CCG GCG GAC TTT C-3′	rat
$p87^{PIKAP}$ reverse	5′-GAC GCC CAG TGA GGC TGT C-3′	rat

To obtain an approximate quantification of the number of mRNA molecules encoding PI3Kγ subunits present in a selection of tissues and cell types, competitive PCR assays were performed. In competitive PCRs, an internal standard is used that is amplified together with the fragment to be quantified. The internal standard is ideally as similar to that fragment as possible to warrant comparable amplification properties for both fragments. Therefore, internal standard templates were generated for p101 and $p87^{PIKAP}$ that have a 40-bp deletion upstream of the 3′-primer binding site but are otherwise identical to the fragments amplified from cDNA samples. Because both internal standard and the fragment in focus are amplified in the same reaction under identical conditions, the number of template molecules for both will be equal if the same amount of product is obtained. The amount of internal standard added to the reaction setup is varied in order to find such conditions of equal PCR efficiency.

The internal standard or competitor constructs were generated by PCR on purified p101 and $p87^{PIKAP}$ fragments from multiplex PCRs (see Table 3.5 for their properties). To introduce the

deletion, modified reverse primers were used in combination with the same forward primers as in the multiplex PCRs (see Table 3.6). As competitor reverse primers, 5'-GTG GGG CTG TCA GTG TAA ATG GCT GCC CCT GGA CC-3' ($p87^{PIKAP}$) and 5'-GCA GAG CCC CAC TGA ATG TCG TCT CTG CTG GCT GG-3' (p101) were used. The PCR products were purified and inspected by agarose gel electrophoresis. Quantification was performed by UV spectroscopy. Serial dilutions of the competitor fragments were used in the competitive PCRs. The intensities of the PCR bands were quantified and analyzed with TINA 2.09 software (Raytest, Straubenhardt) using flatfield-corrected 16-bit image files obtained on the gel documentation system (see above).

Materials for multiplex PCR *Taq* DNA polymerase (Promega, Mannheim), µMACS mRNA kit (Miltenyi Biotec, Bergisch Gladbach), High Pure RNA kit, High Pure PCR product purification kit (Roche Applied Science, Mannheim). For further materials see section 3.2.1.

3.2.4 Northern blot analysis

Northern blot analysis was carried out using premade multiple tissue Northern blots that contained 2 µg poly(A^+) RNA per lane and were prepared from tissues of 8–10-week-old mice. For the generation of radioactively labeled RNA probes, either 1 µl of the supplied linearized β-actin control vector or Bgl II-linearized vector encoding murine $p87^{PIKAP}$-FLAG was used. To prepare linearized $p87^{PIKAP}$-FLAG, 12 µg plasmid was digested for 3 h at 37°C and then precipitated by addition of 1/8th (v/v) 1.5 M sodium acetate and 250 mM EDTA. After incubation for 30 min at -20°C, the DNA was pelleted by centrifugation and resuspended in 10 µl RNase-free water. Of that, 2 µl was used for the labeling reaction, which was performed following the manufacturer's protocol using Sp6 RNA polymerase and 5 µl of labeled UTP (10 mCi/ml stock). After labeling, template DNA was digested with DNase I to prevent it from binding to the RNA probe during hybridization. A ~ 550 bp RNA probe was generated from the linearized $p87^{PIKAP}$-FLAG construct by virtue of the Sp6 promoter in pcDNA3.

Half of the labeling reaction was used for each blot in the hybridization. It was added directly to the membranes which were prehybridized in 10 ml of UltraHyb buffer for 30 min at 68°C. After overnight hybridization, blots were washed 2×5 min with low stringency wash buffer and 2×15 min with high stringency wash buffer. Blots were exposed to an image plate for 18 h. The image plate was read on a phosphoimager (Fujifilm BAS Reader 1500).

Before incubation with the β-actin control probe, hybridized $p87^{PIKAP}$ probe was removed with the probe degradation buffer of the StripEZ kit following the supplied protocol. Hybridization with the β-actin probe was performed as for the $p87^{PIKAP}$ probe. The image plate was exposed for 2 h.

Materials for Northern blot analysis Multiple tissue Northern blots (Clontech, Mountain View, USA), α-[^{32}P]-UTP (NEN *via* Perkin Elmer, Rodgau), StripEZ-SP6 Kit, UltraHyb hybridization buffer, NorthernMax wash buffers (Ambion, Huntingdon, UK), image plate BAS-MS 2040 (Fujifilm *via* Raytest, Straubenhardt). For further materials see section 3.2.1.

3.2.5 *In silico* methods

For the generation of vector maps, evaluation of sequencing data, design of primers, and for the generation of dot-plot alignments the respective programs of the Winstar suite (DNASTAR, Madison, WI, USA) were used.

The p101 homologue p87$^{\text{PIKAP}}$ was identified using the basic local alignment search tool (BLAST; see Altschul *et al.*, 1990; Gish & States, 1993) in the tblastn version, which searches the non-redundant DNA data base for nucleic acid sequences encoding the input protein query. The program was accessed *via* the world wide web at http://www.ncbi.nlm.nih.gov/blast/index.shtml. The default settings and the BLOSUM62 matrix (Henikoff & Henikoff, 1992) were used.

For protein sequence alignments, the CLUSTALW algorithm (Thompson *et al.*, 1994) with the Gonnet matrix (Gonnet *et al.*, 1992) were run and accessed under http://www.ebi.ac.uk/clustalw. The alignments were visualized with the ESPript tool (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi; Gouet *et al.*, 2003).

3.3 Immunochemical assays

3.3.1 Gel electrophoresis and immunoblot

To separate proteins according to their molecular weight, discontinuous sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed according to the protocol established by Laemmli (1970).

In general, samples were prepared by lysing cells directly into 1× SDS sample buffer after treatment with effectors or controls. 200 µl of sample buffer was used per 35-mm dish. Samples were boiled at 95°C for 5 min and then centrifuged for 1 min to collect the sample. If samples were too viscous to pipet, they were sonicated for 5–10 s at 20–30 W (Sonifier B-12 with micro tip, Branson, Danbury, USA). 5–30 µl of sample were loaded per well. SDS-PAGE was performed with 1.5-mm mini gels (Mini-Protean 3 system, Bio-Rad, München) at a constant voltage of 60 V for the stacking and 120–150 V for the separation. Separated proteins were transferred to nitrocellulose membranes by tank-blotting for 90–150 min at a maximum voltage of 110 V and 100 mA (per gel, constant current).

For detection of proteins by Western blot, membranes were washed for 5 min in TBS before they were incubated for 1 h in blocking buffer to saturate unspecific binding sites on the mem-

brane. After a 5 min wash in TBS-T, membranes were incubated overnight with the primary antibody in antibody dilution buffer. Blots were washed thrice for 5 min with TBS-T before incubation with the respective secondary antibody for 1 h at room temperature. Following three washes with TBS-T, bound horseradish peroxidase (HRP)-coupled secondary antibodies were detected *via* a chemiluminescence reaction. Signals were documented using a CCD-based camera system (LAS-1000, Fujifilm *via* Raytest, Straubenhardt). Dilutions of primary and secondary antibodies are given in Table 3.7. The antiserum directed against $p87^{PIKAP}$ was generated in the course of this work by immunizing rabbits with purified His-tagged $p87^{PIKAP}$ (see section 3.5). The immunization of rabbits and the generation of sera was carried out by a commercial service (Pineda Antikörper-Service, Berlin).

Table 3.7: Antibodies

Antibody	Dilution	Generated in	Incubation with	Source
FLAG	1:1000	mouse	dried milk	Sigma, Schnelldorf
GFP (A. v. peptide antibody)	1:1000	rabbit	dried milk	Clontech, Mountain View, USA
Akt	1:1000	rabbit	BSA	Cell Signaling, Frankfurt a. M.
Phospho-Akt (Ser473)	1:1000	rabbit	BSA	Cell Signaling, Frankfurt a. M.
p110γ	1:1000	rabbit	BSA	Cell Signaling, Frankfurt a. M.
$p87^{PIKAP}$	1:2000	rabbit	dried milk	Pineda, Berlin
anti-rabbit IgG (HRP)	1:2000	goat	dried milk	Sigma, Schnelldorf
anti-mouse IgG (HRP)	1:5000	goat	dried milk	Sigma, Schnelldorf

- **1× SDS sample buffer** 63 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.1% bromphenol blue.
- **Running buffer** 25 mM Tris, 192 mM glycine, 0.1% SDS.
- **Stacking gel composition** 125 mM Tris/HCl pH 6.8, 3.9% acrylamide solution, 0.1% SDS. APS and TEMED were added to induce polymerisation prior to casting the gel.
- **Separating gel composition** 375 mM Tris/HCl pH 8.8, 7–12% acrylamide solution, 0.1% SDS. APS and TEMED were added to induce polymerisation prior to casting the gel.
- **Transfer buffer** 25 mM Tris, 0.2 M glycine, 20% methanol or ethanol.
- **TBS** 20 mM Tris/HCl pH 7.6, 137 mM NaCl.
- **TBS-T** 20 mM Tris/HCl pH 7.6, 137 mM NaCl, 0.1% Tween-20.

- **Blocking buffer** 20 mM Tris/HCl pH 7.6, 137 mM NaCl, 0.1% Tween-20, 5% (w/v) nonfat dried milk powder.
- **Antibody dilution buffer** 20 mM Tris/HCl pH 7.6, 137 mM NaCl, 0.1% Tween-20, 5% (w/v) nonfat dried milk powder or 5% (w/v) BSA.

Materials for SDS-PAGE and Western blot Precision Plus Dual Color Protein Standard, TEMED (Bio-Rad, München), Benchmark Pre-Stained Protein Ladder (Invitrogen, Karlsruhe), Hybond ECL nitrocellulose membranes (Amersham Pharmacia, Freiburg), Chemiluminescence Detection kit, glycine, nonfat dried milk powder (Applichem, Darmstadt), 30% acrylamide solution (37.5:1 mono/bis acrylamide, Roth, Karlsruhe), APS, bromphenol blue (Sigma, Schnelldorf).

3.3.2 Immunoprecipitation

Immunoprecipitation (IP) was used to assay protein-protein interactions *in vitro*. One of the interaction partners is bound by a specific antibody, allowing it to be separated from the remainder of the cell lysate by virtue of the antibody-binding properties of protein A-coupled Sepharose. The precipitate is then washed and assayed for the presence of other proteins that have been co-immunoprecipitated through their interaction with the antibody-bound protein.

48 h after transfection, cells were washed with PBS and lysed in ice-cold lysis buffer (600 µl per 60-mm dish). The lysate was rotated for 10 min at 4°C and then centrifuged for 10 min at 12,000×g to pellet unsolubilized cells and debris. The protein of interest was then immunoprecipitated by addition of antibody (4 µg anti-FLAG antibody) and 6 mg protein A Sepharose (preequilibrated in 50 µl lysis buffer) to 500 µl cell lysate. The mixture was rotated overnight at 4°C. After centrifugation, pellets were washed thrice with high-salt lysis buffer and once with lysis buffer and finally resuspended in 100 µl 1× SDS sample buffer. Samples were then subjected to SDS-PAGE and analyzed for the presence of precipitated protein and co-precipitated interacting proteins as described in section 3.3.1.

For some experiments concerning the interaction between $p87^{PIKAP}$ and PDE3B, an alternative IP protocol was used. After washing with PBS, cells were scraped off the plate and collected by centrifugation for 10 min at 100×g. The cells were resuspended in 2 ml ice-cold membrane buffer and lysed by aspiration through a 26-gauge needle. Particulate material was pelleted (15 min, 12,000×g) and resolubilized in 600 µl RIPA buffer. Samples were then processed further as described above.

- **PBS** 8 mM Na_2HPO_4 / 1.5 mM KH_2PO_4 pH 7.4, 128 mM NaCl, 2.7 mM KCl.
- **lysis buffer** 20 mM Tris/HCl pH 7.5, 100 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM

phenylmethylsulfonyl fluoride, 0.5% Igepal Nonidet P-40, 3.2 µg/ml soybean trypsin inhibitor, 0.5 mM benzamidine, 2 µg/ml aprotinin.

- **high-salt lysis buffer** 20 mM Tris/HCl pH 7.5, 1 M NaCl, 50 mM NaF, 1 mM EDTA, 0.5% Igepal Nonidet P-40, protease inhibitors as in lysis buffer.
- **membrane buffer** 50 mM HEPES/NaOH pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 3.2 µg/ml soybean trypsin inhibitor, 0.5 mM benzamidine, 2 µg/ml aprotinin.
- **RIPA buffer** 20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Igepal Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 3.2 µg/ml soybean trypsin inhibitor, 0.5 mM benzamidine, 2 µg/ml aprotinin.

Materials for immunoprecipitation Aprotinin, benzamidine, phenylmethylsulfonyl fluoride, protein A Sepharose, soybean trypsin inhibitor (Sigma, Schnelldorf), Nonidet P-40 (Applichem, Darmstadt).

3.4 Biochemical assays

3.4.1 Phosphodiesterase activity assay

Phosphodiesterases catalyze the breakdown of adenosine 3',5'-cyclic monophosphate (cAMP) to adenosine 5'-monophosphate (AMP). Their activity can be measured by a coupled enzymatic assay that was first described by Thompson and Appleman (1971) and modified to the protocol employed here by Shepherd *et al.* (2004). The PDE activity present in the reaction converts the radioactively labeled cAMP to AMP, which is a substrate for snake venom nucleotidase that further cleaves it to inorganic phosphate and adenosine. The remaining cAMP is separated from the uncharged adenosine by an anion exchange resin. The adenosine is then quantified by liquid scintillation counting.

Cells were lysed and cleared as described in section 3.3.2. Aliquots of the lysates were diluted in reaction buffer to 100 µl final reaction volume and incubated at 30°C for 10 min. Substrate turnover did not exceed 15% of the cAMP present in the reaction, allowing reaction kinetics to be assumed linear and not substrate-limited. Thus, comparability between samples is maintained. The reactions were terminated by boiling the samples for 3 min at 95°C. After cooling on ice, 25 µg of snake venom was added, and the samples were incubated at 30°C for a further 10 min. 400 µl of Dowex suspension was added and the samples were rocked on ice for 20 min before they were centrifuged for 3 min at 12,000×g. The (2,8)-^{3}H-adenosine in the supernatant was then quantified by liquid scintillation counting. A 50-µl aliquot was added to 3 ml of scintillation cocktail.

To specifically assess PDE3 activities, the activity measured in the presence of the PDE3-specific inhibitor cilostamide (10 µM final concentration) was subtracted from the activity measured without PDE inhibitor. The difference in activity was then due to PDEs of the PDE3 family. All samples were assayed in duplicate.

- **reaction buffer** 20 mM Tris/HCl pH 7.4, 5 mM $MgCl_2$, 1 µM unlabeled cAMP, 0.075 µCi/reaction (2,8)-^{3}H-cAMP.
- **snake venom solution** Snake venom was reconstituted in distilled water at 10 mg/ml and diluted 1:10 in 20 mM Tris pH 7.4 prior to adding it to the samples.
- **Dowex suspension** Dowex was washed once in an excess of 1 M NaOH, 5× in distilled water, once in 1 M HCl and 5× in distilled water. The dowex was then stored as a 1:1 dowex/water mixture. Immediately prior to use, one volume of 100% ethanol was added to two volumes of the dowex/water mixture.

Materials for PDE assay (2,8)-^{3}H-adenosine (1 µCi/µl; NEN *via* Perkin-Elmer, Rodgau), BCA protein assay (Pierce, Bonn), cAMP, cilostamide, Dowex (1×8, 200–400), snake venom from *crotalus atrox* (Sigma, Schnelldorf), scintillation cocktail (Zinsser, Frankfurt am Main). For further materials see section 3.3.2.

3.4.2 β-Hexosaminidase assay

RBL-2H3 cells are a common model cell line for mast cells, because they are able to degranulate upon crosslinking of their high affinity FcϵRI receptors by antigen (Oliver *et al.*, 1988). Degranulation was assayed by quantifying the extent of β-hexosaminidase release from intracellular mediator-containing granules to the extracellular medium as described by Ozawa *et al.* (1993).

RBL-2H3 cells were seeded on 24-well plates at 5×10^4 cells per well. Two days after seeding, the medium was exchanged with fresh medium containing 200 ng/ml α-dinitrophenyl (DNP)-BSA antibody. The next day, cells were washed thrice with Hank's buffer to remove excess antibody. Cell were stimulated at 37°C in 500 µl Hank's buffer with antigen (100 ng/ml DNP-BSA) and/or 1 µM IB-MECA. 200 nM ionomycin combined with 50 nM phorbol-myristate-acetate (PMA) served as a positive control, whereas incubation in Hank's buffer without additions was used to quantify basal β-hexosaminidase secretion. All conditions were assayed in triplicate. After 30 min, 20-µl aliquots were transferred to 96-well plates and mixed with 50 µl p-NAG solution and incubated for 1 h at 37°C. Reactions were terminated with 200 µl stop buffer. The alkaline pH of the buffer leads to deprotonation of the released DNP moiety, which then absorbs at 410 nm. Absorption was measured with a microtiter plate reader. Release was calculated as the percentage of the total β-hexosaminidase content, which was calculated as the

sum of the absorptions of lysate of untreated cells (500 µl Hank's buffer containing 1% Triton X-100) and of their basal secretion value.

- **Hank's buffer** 20 mM HEPES/NaOH pH 7.4, 138 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 1 mM $CaCl_2$, 5.5 mM glucose.
- **p-NAG solution** 1 mM 4-nitrophenyl N-acetyl-β-D-glucosaminide (p-NAG) in 100 mM citric acid/sodium citrate pH 4.5.
- **stop buffer** 200 mM $NaHCO_3/Na_2CO_3$ pH 10.

Materials for β-hexosaminidase assay Ionomycin (Alexis, Lausen, Switzerland), DNP-BSA, IB-MECA, monoclonal anti-DNP antibody clone SPE-7, 4-nitrophenyl N-acetyl-β-D-glucosaminide, PMA (Sigma, Schnelldorf).

3.4.3 Peptide SPOT overlay assay

Peptide SPOT arrays are helpful tools in studying protein-protein interactions, protein-DNA interactions, and substrate determinants of kinases (Reineke *et al.*, 2001). Peptides are synthesized on cellulose membranes as spots whose size is defined by the diffusion of the reagents applied to the membrane (Frank, 1992). The technique allows to synthesize and assay large numbers of peptides in comparatively short time. To screen an array of peptides for sequences interacting with a given protein, the membrane is subjected to an overlay assay similar to an immunoblot protocol. Spots of peptides that have bound the applied protein of interest are detected *via* chemiluminescence generated by HRP coupled to an antibody against the applied protein.

Peptide SPOT arrays used in this study were designed in collaboration with the peptide synthesis group at the Leibniz-Institut für Molekulare Pharmakologie. Peptides were synthesized onto β-alanine-functionalized Whatman 50 paper by automated F-moc synthesis using an Auto-Spot robot (ASP222; Invartis AG, Köln) and standard protocols (Kramer & Schneider-Mergener, 1998). Cys residues were replaced by Ser to avoid formation of disulfide bonds between peptides. The free N termini were acetylated to neutralize their charge. The protein sequences to be screened were covered by peptides of 15 and 25 aa length sharing an overlap of 12 and 20 aa, respectively, with the adjacent peptides.

Membranes were prewashed once in methanol for 10 min and thrice in overlay-TBS. Blocking was performed for 3 h at room temperature. After washing the membranes with overlay-TBS for 10 min, they were incubated overnight with 40 µg/ml purified His-tagged protein (see section 3.5) in blocking buffer. Negative controls for direct binding of the antibody to peptides were performed by incubating membranes overnight in blocking buffer only. Membranes

were washed three times in overlay-TBS and subsequently incubated with HRP-coupled anti-His antibody (1:2000 in blocking buffer) for 3 h at room temperature. Following three washes with overlay-TBS, bound HRP-coupled antibodies were detected. Signals were documented as flatfield-corrected 16-bit image files using a CCD-based camera system (LAS-1000, Fujifilm *via* Raytest, Straubenhardt). Images were cropped and background-corrected with a rolling ball algorithm and a ball radius of 40 pixels (ImageJ, http://rsb.info.nih.gov/ij/; NIH, Bethesda, MD, USA). Pixel intensities were integrated over each spot for both the actual overlay and the antibody control. To obtain intensities per aa, the integrated spot signals were divided by the number of aa in the peptide. The aa-normalized intensities were summed for each aa over all peptides that contain the aa in focus. To control for unspecific binding of the detection antibody, the sum derived for the overlay was then divided by the background sum for each aa.

- **overlay-TBS** 50 mM Tris/HCl pH 8.0, 137 mM NaCl, 2.5 mM KCl.
- **blocking buffer** 50 mM Tris/HCl pH 8.0, 137 mM NaCl, 2.5 mM KCl, 10% (v/v) 10× Blocking Buffer, 5% sucrose.

For assays employing purified G$\beta\gamma$, 0.05% decaethylene glycol monododecyl ether (C12E10) was included in all buffers to ensure solubility of G$\beta\gamma$.

Materials for SDS-PAGE and Western blot Anti-His HRP conjugate (Qiagen, Hilden), 10× Blocking Buffer, decaethylene glycol monododecyl ether (Sigma, Schnelldorf), Chemiluminescence Detection kit (Applichem, Darmstadt).

3.5 Protein purification

Spodoptera frugiperda (Sf; fall armyworm) insect cells represent a well-established system for heterologous over-expression of mammalian proteins. In contrast to *E. coli*, insect cells are capable of performing many of the posttranslational modifications that are carried out in mammalian cells and contain a more sophisticated chaperone system as *E. coli*, leading to enhanced procession of mammalian proteins. The insect cell line used here was derived from ovarian cells of *Spodoptera frugiperda* and is called Sf21 (Vaughn *et al.*, 1977). Sf21 cells can be infected by baculoviruses, which are used as vectors to introduce the cDNA of the protein to be expressed into the cells. Expression of the protein of interest at high levels is driven by the baculovirus-derived polyhedrin promoter (Luckow & Summers, 1988).

Materials for Sf21 cell culture and protein purification C12E10, EX-CELL TiterHigh medium, soybean trypsin inhibitor, sodium cholate, N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK; Sigma, Schnelldorf), CellFectin, Sf21 cells (Invitrogen, Karlsruhe), pefabloc SC (Roche

Applied Science, Mannheim), Ni-NTA superflow (Qiagen, Hilden), Resource Q anion exchange resin, Resource S cation exchange resin (Amersham Pharmacia, Freiburg).

3.5.1 Culture and infection of Sf21 cells

Sf21 cells were grown in suspension cultures at 27°C and 75 rpm. EX-CELL TiterHigh medium was used without serum or further supplements. Cells were passaged every 2–3 days by dilution with fresh medium to 0.5–1$\times 10^6$ cells/ml. Cell density was ~4$\times 10^6$ cells/ml at the time of subculturing and was kept below 8–10$\times 10^6$ cells/ml at all times.

For the generation of new baculovirus, cells were seeded in 6-well plates at a density of 4$\times 10^5$ cells per well and allowed to settle. Such monolayers of Sf21 were then transfected with 0.2–1 µg bacmid DNA (see section 3.2.1) using CellFectin. 5 h after transfection, the medium was replaced by 2 ml of fresh medium. Cells were monitored for 5–7 days before the medium was collected and cleared from cellular debris by centrifugation at 1000×g for 15 min. 1 ml of this cleared supernatant was used to inoculate 25 ml of cell suspension (2$\times 10^6$ cells/ml) for 5 days to generate the first virus stock. For subsequent routine amplifications, fresh cells at a density of 2$\times 10^6$ cells/ml were inoculated with 1% (v/v) of the previous stock and cultured for 4–5 days. Cellular debris was removed by centrifugation, and 10% FBS was added to enhance virus stability for long-term storage at 4°C.

For protein expression, 1 l of culture (4$\times 10^6$ cells/ml) was inoculated with 60 ml of virus stock. 48 h after infection, cells were harvested by centrifugation at 200×g for 5 min. If cells were not used for protein purification immediately, pellets were snap-frozen in liquid nitrogen and stored at -80°C.

3.5.2 Purification of PI3Kγ subunits and G$\beta\gamma$ by affinity chromatography

Recombinant PI3Kγ subunits were purified from Sf21 cells using a combination of Ni-NTA affinity chromatography and ion exchange chromatography. According to the specific properties of the proteins, slightly different strategies were employed for each protein. In general, cells were resuspended in homogenization buffer and lysed by 15 strokes at 600 rpm with a glass/teflon homogenizer (B. Braun, Melsungen). Debris was removed by ultracentrifugation for 30 min at 255,000×g in a 60 Ti rotor (Beckman, Krefeld). Ni-NTA chromatography was performed in conventional column systems, while ion exchange chromatography was performed on Resource columns using an Äkta purifier system (Amersham Pharmacia, Freiburg). Purity of proteins was assessed by SDS-PAGE and Coomassie staining of gels.

His-p110γ

In the cleared cytosolic extract, imidazole was added to a final concentration of 10 mM, and the concentration of NaCl was raised to 300 mM. After loading the sample onto a Ni-NTA column,

it was washed with 15 column volumes (CV) buffer W1, 6 CV buffer W2, 1 CV buffer W3, and 1 CV buffer W4 to remove excess salt. His-p110γ was eluted with 6 CV buffer E. The pooled eluate was diluted 1:10 with buffer A and applied to a Resource Q column *via* a superloop. The column was washed with 5 CV buffer A, and protein was eluted with a linear gradient from 0–100% buffer B over 20 CV. Fractions of 0.5 ml were collected. The column was regenerated with 5 CV buffer B.

- **homogenization buffer** 50 mM HEPES/NaOH pH 8, 150 mM NaCl, 10 mM β-ME, 0.2 mM pefabloc SC, 30 µg/ml soybean trypsin inhibitor, 30 µg/ml TPCK.
- **buffer W1** 20 mM HEPES/NaOH pH 8, 300 mM NaCl, 10 mM β-ME.
- **buffer W2** 20 mM HEPES/NaOH pH 8, 300 mM NaCl, 10 mM imidazole, 10 mM β-ME.
- **buffer W3** 20 mM HEPES/NaOH pH 8, 300 mM NaCl, 20 mM imidazole, 10 mM β-ME.
- **buffer W4** 20 mM HEPES/NaOH pH 8, 25 mM NaCl, 10 mM β-ME.
- **buffer E** 20 mM Tris/HCl pH 8.4, 200 mM imidazole, 10 mM β-ME.
- **buffer A** 20 mM Tris/HCl pH 8, 2 mM DTT.
- **buffer B** 20 mM Tris/HCl pH 8, 1 M NaCl, 2 mM DTT.

His-p87$^{\text{PIKAP}}$

The cleared cytosolic extract was diluted 1:2 with buffer A, and His-tagged protein was allowed to bind to Ni-NTA in batch format by rotation at 4°C for 1 h. The resin was loaded to a column and washed by applying 3 CV of buffer B. To remove excess salt interfering with anion exchange chromatography, the column was washed with 2 CV of buffer C before elution with 15 CV of buffer E. The eluted fractions were pooled and diluted 1:2 with buffer A1. The sample was loaded on a Resource Q column. After loading, the column was washed with 5 CV of buffer A1. Then, 1.5 CV of buffer B1 were applied to the column before the flow was stopped for 10 min to allow for equilibration of the column to the lower pH. Fractions of 0.5 ml were collected during the following application of 15 CV of buffer B1.

- **homogenization buffer** 50 mM HEPES/NaOH pH 8, 150 mM NaCl, 10 mM β-ME, 0.2 mM pefabloc SC, 30 µg/ml soybean trypsin inhibitor, 30 µg/ml TPCK.
- **buffer A** 50 mM HEPES/NaOH pH 8, 300 mM NaCl, 30 mM imidazole, 10 mM β-ME.
- **buffer B** 20 mM HEPES/NaOH pH 8, 300 mM NaCl, 30 mM imidazole, 10 mM β-ME.
- **buffer C** 20 mM HEPES/NaOH pH 8, 25 mM NaCl, 10 mM β-ME.

- **buffer E** 20 mM Tris/HCl pH 8.4, 200 mM imidazole, 10 mM β-ME.
- **buffer A1** 20 mM Tris/HCl pH 8.4, 2 mM DTT.
- **buffer B1** 20 mM Tris/HCl pH 7.5, 110 mM NaCl, 2 mM DTT.

His-p110γ/His-p87$^{\text{PIKAP}}$ dimer

Prior to loading onto a Ni-NTA column, the cytosolic extract prepared in homogenization buffer was supplemented with 10 mM imidazole and additional 150 mM NaCl. The Ni-NTA column was washed with 10 CV buffer W1, 10 CV buffer W2, 3 CV buffer W3, and 1 CV buffer W4. The pooled eluates (7 CV buffer E) were diluted 1:8 in buffer A1 and applied to an anion exchange column. After loading, it was washed with 5 CV buffer A. A salt gradient (0–50% buffer B over 20 CV) was used for elution.

- **homogenization buffer** 50 mM HEPES/NaOH pH 8, 150 mM NaCl, 10 mM β-ME, 0.2 mM pefabloc SC, 30 µg/ml soybean trypsin inhibitor, 30 µg/ml TPCK.
- **buffer W1** 20 mM HEPES/NaOH pH 8, 300 mM NaCl, 10 mM β-ME.
- **buffer W2** 20 mM HEPES/NaOH pH 8, 300 mM NaCl, 10 mM imidazole, 10 mM β-ME.
- **buffer W3** 20 mM HEPES/NaOH pH 8, 300 mM NaCl, 20 mM imidazole, 10 mM β-ME.
- **buffer W4** 20 mM HEPES/NaOH pH 8, 25 mM NaCl, 10 mM β-ME.
- **buffer E** 20 mM Tris/HCl pH 8.4, 200 mM imidazole, 10 mM β-ME.
- **buffer A1** 20 mM Tris/HCl pH 8, 2 mM DTT.
- **buffer A** 20 mM Tris/HCl pH 8.
- **buffer B** 20 mM Tris/HCl pH 8, 1 M NaCl.

His-TEV-p110γ/p87$^{\text{PIKAP}}$ dimer

The cleared cytosolic extract in homogenization buffer was adjusted to contain 15 mM imidazole, 300 mM NaCl, and 3% glycerol. The lysate was applied to a Ni-NTA column equilibrated with homogenization buffer. It was washed with 10 CV of buffer W1, 10 CV of buffer W2, and 2 CV of buffer W3. 2 CV of buffer W4 were applied to remove excess salt interfering with downstream cation exchange chromatography. The dimeric complex was eluted with 3 CV buffer E. The pooled eluate was diluted 1:10 with buffer V, and the sample was brought to pH 7 with HCl. For ion exchange chromatography, a Resource S column was used. After loading, the column was washed with 5 CV buffer A. Elution was achieved with a linear gradient of

0–50% buffer B over 20 CV. Fractions of 0.5 ml were collected and immediately supplemented with additional 100 mM NaCl. The column was regenerated with 5 CV buffer B.

- **homogenization buffer** 50 mM HEPES/NaOH pH 8, 150 mM NaCl, 10 mM β-ME, 0.2 mM pefabloc SC, 30 µg/ml soybean trypsin inhibitor, 30 µg/ml TPCK.
- **buffer W1** 50 mM HEPES/NaOH pH 8, 300 mM NaCl, 10 mM β-ME, 3% glycerol.
- **buffer W2** 20 mM HEPES/NaOH pH 8, 300 mM NaCl, 10 mM imidazole, 10 mM β-ME, 3% glycerol.
- **buffer W3** 20 mM HEPES/NaOH pH 8, 300 mM NaCl, 20 mM imidazole, 10 mM β-ME, 3% glycerol.
- **buffer W4** 20 mM HEPES/NaOH pH 8, 75 mM NaCl, 10 mM β-ME, 3% glycerol.
- **buffer E** 20 mM HEPES/NaOH pH 8, 75 mM NaCl, 200 mM imidazole, 10 mM β-ME, 3% glycerol.
- **buffer V** 20 mM HEPES/NaOH pH 7, 75 mM NaCl, 2 mM DTT, 3% glycerol.
- **buffer A** 20 mM HEPES/NaOH pH 7, 75 mM NaCl, 1 mM DTT, 3% glycerol.
- **buffer B** 20 mM HEPES/NaOH pH 7, 1 M NaCl, 1 mM DTT, 3% glycerol.

Purification of G$\beta\gamma$ complexes

Owing to its lipid modification, the purification strategy for G$\beta\gamma$ commenced with the generation of membrane preparations rather than cytosolic extracts. Cells were resuspended in homogenization buffer and disrupted in a nitrogen cavitation homogenizer (Parr Instrument Company, Frankfurt a. M.) at 50 bar and 4°C for 30 min. The resulting material was centrifuged at 800×g for 5 min at 4°C to pellet nuclei and debris. Membranes were obtained from the supernatant by ultracentrifugation (Ti 60 rotor, 30 min, 4°C, 255,000×g). The membrane pellet was resuspended in buffer C (10–20 ml/500 ml culture volume) and homogenized by passing it several times through 22 gauge and then through 26 gauge injection needles. Membrane proteins were solubilized by addition of sodium cholate to a final concentration of 1% (w/v) and incubation at 4°C under constant agitation. The membrane extract was cleared by an additional ultracentrifugation step (Ti 60 rotor, 30 min, 4°C, 255,000×g). After dilution in buffer D (1:5) and addition of imidazole to a final concentration of 10 mM, the sample was applied to a Ni-NTA column. After loading, the column was washed with 5 CV buffer W1 and 3 CV buffer W2. His-tagged G$\beta\gamma$ complexes were eluted with 10 CV buffer E. These were pooled and applied to a Resource Q anion exchange column after a 1:2 dilution in buffer A. The column was washed with 5 CV buffer W2. Elution was performed with a linear gradient from 0–80% buffer B over 10 CV. Fractions of 0.5 ml were collected.

- **homogenization buffer** 50 mM HEPES/NaOH pH 8, 200 mM NaCl, 10 mM β-ME, 0.2 mM pefabloc SC, 30 µg/ml soybean trypsin inhibitor, 30 µg/ml TPCK.
- **buffer C** 50 mM HEPES/NaOH pH 8, 50 mM NaCl, 10 mM β-ME, 0.2 mM pefabloc SC, 30 µg/ml soybean trypsin inhibitor, 30 µg/ml TPCK.
- **buffer D** 20 mM HEPES/NaOH pH 8, 100 mM NaCl, 10 mM β-ME, 0.1% C12E10.
- **buffer W1** 20 mM HEPES/NaOH pH 8, 200 mM NaCl, 20 mM imidazole, 10 mM β-ME, 0.1% C12E10.
- **buffer W2** 20 mM HEPES/NaOH pH 8, 25 mM NaCl, 10 mM β-ME, 0.1% C12E10.
- **buffer E** 20 mM Tris pH 8, 25 mM NaCl, 200 mM imidazole, 10 mM β-ME, 0.1% C12E10.
- **buffer A** 20 mM Tris pH 8, 2 mM DTT, 0.1% C12E10.
- **buffer B** 20 mM Tris pH 8, 1 M NaCl, 2 mM DTT, 0.1% C12E10.

3.6 Imaging techniques

3.6.1 Confocal microscopy

A confocal laser scanning microscope (cLSM) was used to assess the subcellular localization of PI3K signaling proteins and translocating biosensors. Compared to conventional epifluorescence microscopy, confocal imaging achieves enhanced spatial resolution and thinner focal volumes by virtue of a confocal pinhole and a scanning laser. Thereby, the resolution approaches the diffraction limit (Pawley, 1995). By exciting the sample point-by-point with a laser that is focused on a single point rather than on the whole sample, the excitation volume is reduced to a cone centered around the point of interest. An aperture in the conjugate focal plane – the confocal pinhole – precludes light originating from out-of-focus planes from reaching the detector, thereby reducing the volume from which the detected light originates to the focal plane.

Cells for imaging experiments were seeded as described in section 3.1. All experiments were performed at room temperature using HBS buffer. Glass coverslips were mounted in custom-made chambers and placed on the stage of an inverted cLSM (LSM 510 meta, Carl Zeiss, Oberkochen). Either a Plan-Apochromat 63×/1.4 or an αPlan-Fluar 100×/1.45 oil immersion objective were used (all objectives from Carl Zeiss). CFP was excited with the 458-nm line of an argon laser *via* a 458-nm main beam splitter. Emission was passed through a 475-nm long pass filter and recorded with the conventional detector of the LSM. If both CFP and YFP were imaged simultaneously, CFP was excited at 458 nm and detected filtered through a CFP-selective band pass filter (470–500 nm; all filters and mirrors from Chroma, Brattleboro, VT,

USA). YFP was excited by the 488-nm line of the argon laser. Emission signals were passed through a 505-nm long pass filter. Pinholes were adjusted to yield optical sections of 0.8–1.4 µm.

- **HBS buffer** 10 mM HEPES/NaOH pH 7.4, 128 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM glucose, 0.2% BSA.

3.6.2 Imaging of intracellular calcium concentrations

The use of calcium-sensitive dyes provides a convenient method to monitor the intracellular concentration of calcium ions ($[Ca^{2+}]_i$) in living cells. Binding of calcium ions to the dye fura-2 (Grynkiewicz *et al.*, 1985) is accompanied by a conformational change in the fura-2 molecule, resulting in a blue-shift of its excitation spectrum. Using spectral fingerprinting and deconvolution methods, signals of both calcium-free and calcium-bound forms of fura-2 as well as signals from fluorescent proteins can be separated. $[Ca^{2+}]_i$ can be calculated using the mass action law and the dissociation constant of the fura-2/Ca^{2+} complex as

$$[Ca^{2+}]_i = 224\,\mathrm{nM} \times \frac{[\mathrm{Fura}_{\mathrm{Ca\text{-}bound}}]}{[\mathrm{Fura}_{\mathrm{Ca\text{-}free}}]} \quad .$$

The epifluorescence microscope used for calcium imaging consisted of an inverted microscope (Axiovert 100; Carl Zeiss) with an 40×/1.3 F-Fluar objective. Monochromatic light for excitation was derived from a xenon lamp equipped with a monochromator (Polychrome IV system; TILL Photonics, Martinsried). A 505-nm dichroic mirror was used as the beam splitter. Emitted light was passed through a 510-nm long pass filter and detected by a cooled CCD camera (Imago, TILL Photonics).

The cells were loaded with the membrane-permeable precursor fura-2/AM (2 µM; Molecular Probes *via* Invitrogen, Karlsruhe) in HBS buffer (see above) for 30 min at 37°C, resulting in intracellular accumulation accompanied by de-esterification to fura-2. The glass coverslips were placed in custom-made chambers for imaging. Cells were sequentially imaged at 340, 380, 430, and 450 nm at a frequency of 1 Hz. The fluorescence signals were recorded separately for each excitation wavelength. Regions of interest were defined for single cells. Cells were washed away at the end of the measurements, and the remaining fluorescence was taken as the background. Based on reference spectra for fluorescent proteins, calcium-free, and calcium-bound fura-2, fluorescence signals of these dyes were separated by multivariate linear regression analysis using a custom-made software (Lenz *et al.*, 2002).

3.6.3 Fluorescence resonance energy transfer

Fluorescence resonance energy transfer (FRET) is a non-radiative transfer of excitation energy from an excited donor fluorophore to an acceptor fluorophore. The quantum mechanical theory of the process was derived by Förster (1948). Accordingly, for efficient dipole-dipole coupling

to occur, the emission spectrum of the donor and the excitation spectrum of the acceptor must overlap. Additionally, both fluorophores have to be in close proximity and in a suitable relative orientation, *i.e.* their transition dipole moments, which generally lie in the planes of the conjugated ring systems, may not be perpendicular to each other. Like fluorescence, FRET is a relaxation pathway for the excited state of the donor fluorophore. Because both deexcitation pathways compete, the fluorescence intensity of the donor is reduced if FRET occurs. The Förster equation describes the dependence of the FRET efficiency E on the molecule distance as

$$E = \left(1 + \left(\frac{r}{R_0}\right)^6\right)^{-1} \quad ,$$

where r is the donor–acceptor distance and R_0 is the Förster radius. The Förster radius is a complex term describing various properties of a donor-acceptor fluorophore pair, such as the spectral overlap and the donor quantum yield. It equals the distance at which half of the excited donor molecules relax *via* FRET. Because of the exponential decay of the FRET efficiency with the distance r, the distance regime that allows FRET is very narrow. Therefore – given that the orientation of the donor and the acceptor is not deleterious – FRET may be used to measure distances on the ångstrom scale (Stryer, 1978).

In combination with fluorescent labels, FRET allows the analysis of protein-protein interactions in living cells. Autofluorescent proteins such as the green fluorescent protein (GFP; Tsien, 1998) and its color variants may be fused to proteins by genetic means. CFP is a commonly used donor fluorophore, while YFP is used as an acceptor (Heim & Tsien, 1996). Their Förster radius is about 5–6 nm. Accordingly, if FRET occurs between CFP and YFP, they have to be closer than 10–12 nm. Such distances, however, are indicative of a direct interaction of the fusion proteins.

To quantify FRET efficiencies, the following equation was used (Cantor & Schimmel, 1980)

$$E = 1 - \left(\frac{F_{DA}}{F_D}\right) \quad ,$$

where F_{DA} is the fluorescence of the donor in the presence of an acceptor and F_D denotes the fluorescence of the donor in the absence of an acceptor. Using an acceptor photobleach protocol, these two situations can be generated from a single sample (Kenworthy, 2001). First, F_{DA} is measured by exciting the donor in the presence of the acceptor. Then, the acceptor is selectively and irreversibly bleached by strong illumination at its excitation maximum. F_D is calculated from a linear regression of the increase in donor fluorescence with the decrease in acceptor fluorescence and extrapolation to zero acceptor fluorescence (Hofmann *et al.*, 2002).

The same epifluorescence microscope as described in the previous section was used to image CFP and YFP for FRET microscopy with the following adaptations. The beam splitter was a dual reflectivity dichroic mirror (< 460 nm and 500–520 nm), and the emission of CFP and YFP

was separated by suitable band pass filters (475–505 nm for CFP and 535–565 nm for YFP), which were exchanged by a motorized filter wheel (Lambda 10/2; Sutter Instruments, Novato, CA, USA). In each image acquisition cycle, CFP and YFP were excited at 410 and 515 nm for 40 and 8 ms, respectively. Images were taken every 2 s. After 15 cycles, YFP was bleached by an additional 1.8-s illumination at 512 nm for 60 cycles, typically resulting in ~95% YFP photobleach.