Chapter 2

MATERIALS

(for all Chapters)

Antibodies.

- Rabbit polyclonal anti-pan p85 antibody (recognized N-terminal SH2 domain), gift from Cantley lab otherwise commercially available: Upstate Biotechnology, NY
- Mouse monoclonal anti-p55gamma antibody, gift from M. White
- Anti-p110alpha monoclonal antibody (P94520), Transduction laboratories
- Rabbit polyclonal anti-p110alpha antibody (SC-7174), Santa Cruz
- Rabbit polyclonal anti-p110beta antibody (SC-602), Santa Cruz
- Rabbit polyclonal anti-Akt antibody (9272), rabbit polyclonal anti-MAPK antibody (9102), or rabbit polyclonal antibodies for the phosphorylated proteins (9271L, 9101S), Cell signaling, MA
- Mouse monoclonal anti-phosphotyrosine antibody (4G10) was a gift from T. Roberts
- Mouse monoclonal anti-phosphotyrosine antibody (P100), Cell signaling
- Anti-PDGF receptor antibody, gift from Neel lab
- Anti-IR antibody, gift from Kahn lab
- Anti-IRS1 antibody, gift from Kahn lab
- Anti-IRS2 antibody, gift from Kahn lab
- Goat polyclonal anti-Akt antibody (N19), Santa Cruz
- Goat anti-rabbit IgG, HRP conjugate, chemicon
- Goat anti-mouse IgG, HRP conjugate, chemicon
- Goat anti-rabbit IgG, Texas red conjugate, Jackson Immuno Laboratories
- Goat anti-rabbit IgG, FITC conjugate, Jackson Immuno Laboratories
- Anti-mouse IgM, Texas red conjugate, Jackson Immuno Laboratories

Growth Factors and Lipids.

- Platelet derived growth factor: PDGF-BB human recombinant, Austral Biologicals
- Platelet derived growth factor: PDGF-AA human recombinant, Sigmal
- Insulin: Lilly Research Laboratories, Indianapolis
- Insulin like growth factor 1: IGF-1human recombinant, Austral Biologicals
- Epidermal growth factor: EGF human recombinant, Upstate Technologies
- Lysophosphatidic acid, Sigma
- Human fibronectin, Becton Dickinson
- PS, PI and PI-4,5-P_2, Avanti Polar Lipids
- PI-4-P, Sigma

Inhibitors.

- Wortmannin, Sigma
- LY294002, Calbiochem

Expression plasmids.

- pMIG retroviral vector: Murine Stem Cell Virus IRES GFP
- pMIG-p85alpha cDNA of murine p85alpha
- pMIG-p85beta cDNA of murine p85beta
- pMIG-p50alpha cDNA of murine p50alpha
- pCMV5-VAV2 cDNA of murine Vav2
- pBSSK-p85beta cDNA of murine p85beta
- pGEX4T prokaryotic GST gene fusion vector
- pGEX4T-SH3p85alpha cDNA of SH3 domain of murine p85alpha
- pGEX4T-SH3p85beta cDNA of SH3 domain of murine p85beta

Bacteria.

- DH5alpha (E.coli)

Primers.

- P110alpha forward (2): 5'TGG CAT CCT CAA ATG ATA GTA ACA 3'
- P110alpha reverse: 5'GAA TAA AAT AAA GAG GAG GCA TCA TAG 3'
- P110beta forward: 5'GTA TTT GGA CCT GAT TTG ATG ATA G 3'
- P110beta reverse: 5'GTT TTT ATT TGA AAT TAA ATC ACA TCG C 3'
- Neomycin: 5'GAA GGC TCT TTA CTA TTG CTT TAT 3'
- SH3 domain of murine p85beta: 5'TCA TCA GAA TTC TCA GGG TCC TAG GAA CTC CAC 3' and 5'ACA ACA GGA TCC GCA GGA GCC GAG GGC TTC 3'

Other reagents and materials.

1. Cell culture:

- Cell culture medium: DMEM, Bio Whittaker
- FCS: fetal bovine calf serum: HYCLONE
- Antibiotics (penicillin, streptomycin): 100U/ml, GIBCO BRL
- L-glutamine, GIBCO BRL
- PBS (phosphate buffered saline): 150mM NaCl, 3mM KCl, 10Mm Na_2HPO_4, 2mM KH_2PO_4 (pH7.4)
- Trypsin: Bio Whittaker
- 0.22um pore filter: Coring Costar

2. Protein Chemistry:

- Lysis buffer: 25mM Tris/HCl (pH7.2), 137mM NaCl, 10% glycerol, 1% NP-40, 25mM NaF, 10mM $Na_4P_2O_7$, 1mM EDTA
- Protease Inhibitors: AEBSF 4ug/ml, leupeptin 4ug/ml, aproteinin 4ug/ml, pepstatin 4ug/ml
- Protein A sepharose beads, Pharmacia
- Protein G sepharose beads, Amersham Pharmacia
- Centrifuge: MicroCentrifuge VS-15, Shelton Scientific,
- Centrifuge: Centrifuge 5415D, Eppendorf
- 6x sample buffer: 1M Tris/HCl (pH6.8) 10ml, glycerol 20ml,

betamercaptoehtanol 10ml, SDS 4.8g, bromphenol blue 2ml, water 6ml

- Rainbow molecular weight markers, Amersham Pharmacia
- Heat block, Fisher Scientific
- Stacking gel: 4x buffer (Tris/HCl/SDS (pH6.8) (0.5M Tris/HCl (pH6.8) containing 0.4% SDS) 30% acryl amid / 0.8% bisacrylamide), 0.07% TEMED (Sigma), ammonium persulfate
- Separating gel: 4x buffer (Tris/HCl/SDS (pH8.8) (1.5M Tris/HCl (pH8.8) containing 0.4% SDS) 30% acryl amid / 0.8% bisacrylamide), 0.01% TEMED, ammonium persulfate
- SDS-running buffer: 25mM Tris/HCl (pH8.3), 250mM glycine, 0.1% SDS
- Electrophoresis unit, Hoefer
- Power supply: Electrophoresis System, Fisher Biotech
- Filter paper, Whatman
- Nitrocellulose membrane, BioRad laboratories
- Immoblion P (PVDF) membrane, NEN
- Transfer buffer: 39mM glycine, 48mM Tris base, 0.037% SDS, 20% methanol
- Transfer unit: Trans-Blot Cell, BioRad
- Power supply: Power Pac 200, BioRad
- Shaker, Marsh Biomedical Products, Inc
- TBST: 10mM Tris (pH8), 0.15M NaCl, 0.1% Tween-20
- ECL™: NEN™, Life Science Products
- Li-Cor, Odyssey
- Stripping solution: Re-Blot Plus, Chemicon
- TNE: 10mM Tris (pH 7.5), 100mM NaCl, 1mM EDTA
- Phosphoimager, Molecular dynamics
- Coomassie:15% methanol, 10% glacial acetic acid, 2-4g Coomassie Brilliant Blue/ 10 liter total
- Destain solution: 15% methanol, 10% glacial acetic acid
- Partisphere SAX column, Whatman

3. Molecular biology:

- Transfection Reagent: SuperFect Transfection Reagent, QIAGEN
- Polybrene, Sigma
- LB Broth / Lennox: DIFCO Laboratories
- Ampicillin, Sigma
- IPTG, Sigma
- STE: 150mM NaCl, 10mM Tris/Hcl (pH8), 1mM EDTA
- Glutathione Sepharose 4B, Amersham Pharmacia
- QIAprep Spin Miniprep Kit, Qiagen
- Spectrophotometer: Beckman DU 640
- Restriction enzymes / alkaline phosphatase / T4 DNA ligase / Deep vent DNA polymerase, New England Biolabs
- Pulse Controller Plus and Gene Pulser II, BioRad
- Tail digestion buffer: 50mM Tris/HCL (pH8), 1% SDS, 0.1M NaCl, 1mg/ml proteinase K, 100mM EDTA (pH8),
- dNTPs, Roche
- Qiagen Hot start Taq polymerase, Qiagen
- 1kB DNA ladder: GIBCO BRL
- 6x DNA Loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll (Type 400, Pharmacia) in water
- 50x TAE: 242g Tris/HCl, 57.1ml glacial acetic acid, 100ml 0.5M EDTA (pH8) for 1l solution
- electrophoresis chamber: Easy Cast TM Electrophoresis System, Model # B1A, Owl, Scientific, Inc.
- Power supply: Power Pac 1000, BioRad
- Procise 494 Automated Protein Sequencer, Applied Biosystems

4. Cell biological assays:

- Coverslips: micro cover glasses 25mm circle, VWR Scientific, Inc.
- Rhodamine-phalloidin, Molecular Probes
- Flouromount-G slide mounting media, Fisher

- CBS buffer: 10mM MES (pH6.1), 138mM KCl, 3mM $MgCl_2$, 2mM EGTA, 10-11% sucrose
- Time-lapse buffer: DMEM clear media (high glucose, 25mM HEPES), GIBCO BRL
- Microscope: Nikon, DIAPHOT 300
- Imagine program: Image Pro Plus

5. Physiological Studies:

- Tweezers: Dumont, Pattern No. 5 and 55, Biomedical Research
- Fixatives: Bouin's Solution and Formalin Solution, Sigma
- Microscope: Olympus SZX-ILLB100
- TUNEL kit: In situ cell death detection kit – POD or TMR red, Roche
- DAB substrate, Roche
- Rodent chow, Purina
- High fat diet: Adjusted Calories Diet, Haarlen Teklas
- Glucose monitor: One Touch II, Lifescan, Mountain View, CA
- Glucose test strips, Allegiance Health Care
- ELISA KIT, Crystal Chem, Chicago
- Heparinized micro hematocrit capillary tubes, Fisher Scientific
- Buffer A: 25mM Tris/HCl (pH 7.4), 10mM EDTA, 10mM EGTA, 10Mm Na_3VO_4, 50mM $Na_4P_2O_7$, 100mM NaF, 5□g/ml leupeptin, 5□g/ml aprotinin, 2mM PMSF, and 1% (vol/vol) NP-40

EXPERIMENTAL PROCEDURES

1.Cell culture

Tissue cell culture: MEFs were generated from embryos at various developmental stages (E8.5 for p85a-/-p55a-/-p50a-/-p85b-/- and littermate controls [1], and E11.5 for p85a-/-p55a+/+p50a+/+ p85b-/- and littermate controls [2]). The embryos were removed from the deciduas under the microscope and placed into phosphate buffered saline (PBS) containing 15% FCS (if < E9), penicillin and streptomycin. The yolk sac and amnion were removed and the embryos were transferred onto gelatinized (2% gelatin in PBS for 15min at RT) 12 well [1] or 6 well [2] tissue culture dishes containing DMEM with 15% FCS, penicillin, streptomycin and L-glutamine. After a few days, the E8.5 old embryos attached to the plates. The cells started to migrate away from the embryo and covered the whole plate. Beating cardiomyocytes were observed; however, these cells did not survive long term passage. In contrast, in order to remove differentiated cells the head and heart of E11.5 old embryos were removed with foreceps. Then, the embryos were minced with razor plates and trypsinized for 10min before plating. The cells were expanded very slowly (1:2 or 1:3). The growth media was not changed very often in order to not dilute autocrine factors.

Immortalization of primary fibroblasts. The primary embryonic fibroblasts were plated onto gelatinized plates at subconfluent density since retroviral integration is dependent on active cell proliferation. The cells were infected with 2ml culture supernatant derived from a ZIP virus packaging cell line that produces a replication-defective retrovirus expressing SV40 large T antigen (amounts for 10cm dish). To the 2ml viral supernatant, 2ml DMEM and polybrene at a final concentration of 8µg/ml was added. The mixture was incubated for 3h at 37°C. After the incubation, fresh growth media was added. The cells were split the next day. After some passages colonies of immortalized cells were seen. These cells grew more tightly and more homogenously than primary cells. The cells were used for experiments when the infected cells had completely replaced the primary cells. This was the case when the cells in the dishes showed a homogenous pattern at confluency.

Retroviral addback. p85a-/-p55a-/-p50a-/-p85b-/- MEFs were infected with p85a, p85b or p50a in MSCV IRES GFP (pMIG) or pMIG alone. The constructs were a gift from

David Fruman. PT67 cells were seeded into 10cm dishes on day prior to transfection. The next day in the morning, the cells were transfected at a density of 80% via SuperFect transfection kit with either 4ug pMIG, pMIG-p85alpha, pMIG-p85beta or pMIG-p50alpha. After 3hrs, growth media was added to the cells to cause recovery from the transfection procedure over night. The next day in the morning, successful transfection was confirmed by visualizing the GFP caused fluorescence. The cells were washed once with PBS and then the growth media was replaced by 5ml DMEM to cause high titer virus production in the absence of complement factors. 6 and 12h later, the supernatant was collected and spun at 1,000 rpm for 10min at 4°C, in order to remove virus producing cells. The supernatant was sterilized by passing it through a 0,45µm filter. Polybrene was added to a final concentration of 4µg/ml and the whole mixture was incubated overnight with subconfluent (50-80%) p85a/p55a/p50a/p85b-/- MEFs seeded a day before onto a 10cm dish. The next day, cells were washed and growth media was added to cause cell proliferation and hence retroviral integration. The GFP expressing cells were separated from non-infected cells via FACS (BIDMC / Boston) after having been expanded to a 15cm dish.

Transient transfections: Vav2 was introduced into p85a-/-p55a-/-p50a-/-p85b-/- cells by transient transfection using SuperFect protocol according to the manufacturer´s manual.

2. Protein chemistry

Immunoblot. Subconfluent cells were starved 24h and then stimulated with various concentrations of PDGF-BB, IGF-1 or EGF for indicated times (+/- pre-treatment with 100nM wortmannin for 20-30min). Cells were washed three times with ice cold PBS and then lysed in lysis buffer containing fresh protease/phosphatase inhibitors for 10min at 4°C. The clarified lysates were standardized by Bradford assay. 6x samples buffer was diluted to 1x in the lysate, mixed and boiled for 10min at 100°C.

Separation of the proteins. The samples were subjected to SDS-PAGE with the appropriate percentage of polyacrylamide (8% in order to separate p85alpha from p85beta). SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed corresponding to Laemmli (Laemmli, 1970). The proteins were separated by running through the stacking gel at 40mA/gel and the separating gel at 80mA/gel.

Western blot. The gels were transferred onto a 0.2µm nitrocellulose or PVDF membrane using a wet transfer system at 1A for 1h/gel at 4°C in transfer buffer. After the transfer was complete the membrane was rinsed once in TBST. The transfer and the even loading was confirmed via short staining with Panceau S by gentle rocking. The dye was washed away by rinsing a few times in TBST. Non-specific binding sites on the nitrocellulose membrane were blocked with 5% milk or 5% BSA in TBST for 1h at RT or longer at 4°C by gentle rocking. The membrane was rinsed in TBST to remove the remains of blocking reagent.

Incubation with antibodies. The primary antibody was diluted to the appropriate concentration (1/200 – 1/4000, mostly 1/1000) in TBST containing 5% BSA (more rarely 5% milk) to a total volume of about 5-20ml. The antibody was either incubated 1-3h at RT or overnight at 4°C by gentle rocking. After removing the antibody the membrane was rinsed once with TBST, then washed once for 15min and then twice for 5min with TBST (no certain volume) by gentle rocking. The secondary antibody coupled to horseradish peroxidase or a green or red fluorophore was diluted 1/10.000 in TBST containing 5% milk to a total volume of 10-50ml and incubated for 30-60min at RT by gentle rocking. After removing the secondary antibody the same washing procedure as described above was repeated.

Detection via ECL™. A commercial ECL kit (ECL™: NEN™, Life Science Products) was used to develop the western blots. The solutions contain the substrates (hydrogen peroxide and luminal) for the peroxidase that is coupled to the secondary antibody. During the oxidation of luminal via hydrogen peroxide measurable chemiluminescence is produced which can be detected by a photosensitive film.

Detection via ECL™. In case the fluorophore coupled secondary antibodies were used in the hybridization step, the Li-Cor (Odyssey) system was used for detection.

Stripping. In order to reprobe blots for example to compare the amounts of phosphorylated to the unphosphorylated form of this protein, the primary and secondary antibodies were stripped away. The blots were incubated with stripping buffer for 10-30min at RT by gentle rocking and then washed twice with 50ml TBST with 5% milk for 5min at RT. After this procedure the blots were incubated with the primary antibody of interest.

PI3K assay. Equal amounts of clarified cell lysate were immunoprecipitated with anti-p85pan, anti-p110alpha, anti-p110beta or 4G10. The immunoprecipitates were washed two times with lysis buffer and twice with TNE (10mM Tris [pH 7.5], 100mM NaCl, 1mM EDTA). The PI3K assay was performed by adding 20µl HEPES (100mM, pH7), 20µl lipids (67µM lipid substrate and 133µM phosphatidylserine) and 10µl ATP mix (70mM HEPES (pH7), 50mM $MgCl_2$, 0.5mM ATP, $^{\beta 2}$ P-ATP 10uCi / assay) to 50µl of IP. The reactions were performed at room temperature and stopped after 10-20min by adding 25µl 5M HCl. The lipids were extracted with 160µl CHCl3:MeOH (1:1). The phosphorylated lipids were spotted on a TLC and separated over night with 1-propanol : 2M acetic acid (65:35). The radioactivity was visualized by phosphoimager.

HPLC analysis. Subconfluent cells were starved over night and incubated for 45min in phosphate free medium and then labeled for 4h with ^{32}P (2mCi/ 10cm plate). The cells were stimulated with 10ng/ml PDGF for 5min, +/- pre-treatment with 100nM WM for 20-30min. The reaction was stopped with ice-cold PBS. The cells were lysed in 400µl MeOH and 400µl 1M HCl. The lipids were extracted with 400µl $CHCl_3$ and then with 400µl MeOH / 0.1M EDTA. Lipids were deacylated with 1ml methylamine reagent (26.8 ml 40% methylamine in H_20, 16ml H_20, 45.7ml methanol, 11.4 ml n-butanol) for 50min at 53°C. Deacylated lipids were dryed and resuspended in 600µl H_20 and extracted with 600µl n-butanol : petroleumether : ethylformate (20:4:1). Then the lipids were mixed with ^{3}H-labeled standards and analyzed by anion-exchange high performance liquid chromatography (HPLC) using a Partisphere SAX column (Whatman) as described previously (Serunian, Cantley, 1991).

Rac activation assay. The levels of Rac GTP were measured by affinity precipitation using GST-CRIB (Cdc42 and Rac interactive region) of PAK65 (Manser et al., 1994), as previously described (Scita et al., 1999).

In Vitro Akt Kinase Assays. Tissue homogenates were immunoprecipitated with goat anti-Akt antibody and an Akt kinase assay with crosstide as a substrate was performed. The immunoprecipitates were washed and resuspended in 50mM Tris/HCl (pH 7.5), 10mM $MgCl_2$, 1mM DTT to which 20□M ATP, 5 □Ci [□-32P]ATP, and 5□g of crosstide for Akt assay had been added. The reaction was performed at 30°C, after 20min the

reaction was stopped, and the aliquots were spotted onto P-81 paper, washed with 0.5% of phosphoric acid, and counted for radioactivity.

GST-pulldown. Cell lysates (containing DTT of a final concentration of 1µM) of exponentially growing p85a/p55a/p50a/p85b-/- MEFs were prepared as described above.70mg of total cell lysate (1mg/ml) was precleared with 1mg GST-sepharose beads for 2h at 4°C by gentle rocking. Then, the supernatant was split into 3 conical tubes containing each 22ml total cell lysate. 220µg sepharose beads preabsorbed with either GST, GST-SH3p85alpha or GST-SH3p85beta were added (1/100) and incubated for 3h at 4°C by gentle rocking. The beads were then washed three times with 10ml lysis buffer + protease inhibitors + freshly added DTT (1µM final). The interaction partners were eluted off the beads by boiling the samples in 1x sample buffer (final concentration) for 10min at 100°C. However, the doubly precleared supernatants (20mg in 20ml) were again incubated with beads (1/100, so 200µg), but this time the GST-SH3p85alpha or GST-SH3p85beta beads were switched: for example the lysate that had been cleared sequentially with GST and then with GST-SH3p85alpha was now incubated with GST-SH3p85beta for 3h at 4°C by gentle rocking. By doing a reversed incubation, only the binding partners specific for the last SH3 domain used would be pulled out. The beads were then washed three times with 10ml lysis buffer + protease inhibitors + freshly added DTT (1µM final). The interaction partners were eluted off the beads by boiling the samples in 1x sample buffer (final concentration) for 10min at 100°C.

Coomassie stain of proteins. Proteins separated by SDS PAGE were visualized by coomassie stain. The gel was therefore incubated with the preheated dye for 1h by gentle rocking. The gel was then destained for 3d by gentle rocking in destain solution at RT.

Mass spectrometry. Proteins separated by SDS PAGE and visualized by coomassie stain were cut out from the gel and identified by mass spectrometry in the Taplin Biological Mass Spectrometry Facility, Department of Cell Biology, Harvard Medical School. The peptides are analyzed by ion trap mass spectrometry and the amino acid sequence is identified by tandem mass spectrometry (MS/MS) and subsequent database searching.

3. Molecular biology.

Genotyping of mice. Mice were genotyped by PCR of crude tail genomic DNA. 50ul tail buffer without proteinase K was added to the 1mm long tails and heated to 100°C for 10min. The samples were cooled on ice and then proteinase K was added. The tails were digested for 3h at 55°C with gentle rocking. The PCR reaction volume used was 50uL containing 0.25µM of each primer, 4x 200µM dNTPs, 0.125µL of Qiagen Hot Start Taq polymerase and 1x accompanying buffer. The PCR cycle was designed the following: 1. step: 95°C for 15min, 2. step: 95°C for 30sec, 3. step: 57°C for 30sec, 4. step: 72°C for 2min, 5. step 72°C for 10min. Step 2 to 4 was repeated 39 times. The PCR products were visualized on a 2% 1X TAE agarose gel by ethidium bromide staining.

Constructing the GST-Fusion protein of the SH3 domain of p85beta. (a) *Determination of domain boundaries.* The borders of the SH3 domain of p85beta were identified by alignment of p85 isoforms and homologues. The various sequences were found in the NCBI database, edited to the necessary format (gcgpileup) in "Edit Sequence" and then aligned by clustal in "Meg Align". Finally, the degree of homology was analyzed with "Seq vu". The drop of similarity and the cumulative occurrence of aminoacids, such as proline, glycine, serine, aspartate, asparagine was considered to mark the boundaries of the domain.

(b) Amplification of the cDNA coding for the SH3 domain of p85beta by PCR. The cDNA encoding the SH3 domain of p85beta was amplified from the pBSSK-p85beta plasmid by PCR with primers containing either a BamHI restriction site (5'-primer) or an EcoRI restriction site (3'-primer). In order to assure high fidelity "Deep Vent" DNA polymerase was used. The total reaction volume was 50ul containing various concentrations of DNA (10-200ng). The final primer and dNTP concentration was 1uM each and 200uM each, respectively. The PCR cycle was as follows: 1. step: 94°C for 3min, 2. step: 94°C for 30sec, 3. step: 56°C for 30sec, 4. step: 72°C for 45sec, 5. step: 72°C for 5min. Step 2-4 was repeated 29 times.

Subcloning of the cDNA coding for the SH3 domain of p85 into a prokaryotic GST-fusion protein vector. After confirmation of the right PCR product (240bp) on a 2% TAE agarose gel, the fragment was purified with the "Qiaqick PCR" kit and eluted into 30ul H_20. The ends were digested with BamHI (10U) and EcoRI (10U) in EcoRI buffer

containing BSA for 2h at 37°C in a total volume of 50µl. (The primers attaching the restriction sites to domain borders had been designed to end with a short clamp flanking each restriction site to promote the digestion.) The restriction enzymes were inactivated by heating the digest up to 80°C for 20min and removed via the "Mermaid geneclean" kit that is especially designed to achieve a good yield for small DNA fragments. The fragment was then ligated (1µl = 400U T4 DNA ligase) over night at 16°C into the pGEX-4T-3 vector ("vector + insert") or the vector was mmediate without adding the fragment to the reaction ("vector only"). The target vector (5µg) was also digested as described before with BamHI and EcoRI to obtain compatible, sticky ends of the vector and insert. In contrast to the insert, after the inactivation of the restriction enzymes, the vector was treated with alkaline phosphatase (10U) for 1h at 37°C to prevent intramolecular ligation. This reaction was also terminated by heat inactivation for 20min at 80°C. Then, the DNA was precipitated with 2.5 volume 100% Ethanol, 1/10 volume 3M sodiumacetate (pH5.2), 1/10 volume glycogen as carrier for 15min at RT. The precipitation was spun down at 15,000rpm for 15min at RT. The pellet was washed with 70% ethanol and then dried at RT. The DNA was resuspended in 10µl H_20. 5ul of the purified DNA was electroporated into 25-50µl competent DH5alpha bacteria. The bacteria were recovered by rotating them in an eppendorf tube with 1ml LB for 1h at 37°C. Then, various amounts were plated on LB plates containing 50µg/ml ampicillin. The plates were incubated upside down overnight at 37°C in the dark. The next day the success of the integration was determined by comparison of the number of colonies obtained from the ligation "vector + insert" to "vector only". Six colonies from the plate derived from the ligation "vector + insert" were picked into 5ml LB/AMP and simultaneously streaked onto a master plate with LB/AMP. The bacteria were grown over night at 37°C. The next day minipreps was performed using the "Qiagen Miniprep Spin" kit according to the manufacturer's protocol. The purified plasmids (10µl/50µl digest) were verified with a double digest using the restriction enzymes BamHI/EcoRI as described above. Now we could easily use the colonies from the master plate to grow up a bigger culture of pGEX-4T-SH3p85beta and also prepare a glycerol stock by freezing 1ml culture with 1ml 80% sterile glycerol. The stock was stored at –80°C.

Preparing of GST-Fusion proteins. A 100ml culture of LB containing 50µg/ml ampicillin (AMP) was inoculated with either pGEX4T, pGEX4T-SH3p85alpha or pGEX4T-SH3p85beta and grown overnight at 37°C. The next day, the starter culture was diluted 1/10 into 1l LB/AMP and grown at 37°C to a density of OD_{600}=0.6-0.8. The bacteria were induced with 0.4mM (IPTG) and grown for 4h at 37°C. The culture was spun down at 6,000 rpm at 4°C and the pellet was frozen in liquid nitrogen. The bacteria were stored at –80°C until further use. The pellet was thawed on ice and resuspended in 30ml STE containing protease inhibitors. Then, 0.5mg/ml lysozyme was added and the suspension was gently rocked at 4°C. 20mg Sodiumdesoxycholate was added and the solution was rocked for 15min at 4°C. $MgCl_2$ and Dnase were added to a final concentration of 10mM and 10ug/ml, respectively, and the solution was incubated for 15min at 4°C. The bacterial lysate was spun at 15,000rpm for 30min at 4°C. The supernatant was transferred to a conical tube and incubated with 225ul of a 1:1 GSH sepharose slurry for 30-60min at 4°C. The beads were spun down at 1,000rpm for 2min at 4°C and then washed three times with PBS, 0.5% NP-40 + protease inhibitors for 10min. Then, one wash with PBS + protease inhibitors was performed. The beads were stored in PBS containing 25% glycerol at 4°C for one week or at –80°C for long term storage.

Peptide library screen. *(a) Peptide library design*: Two soluble, oriented peptide libraries (MAXXRXXPXXPXXXAKKK and MAXXXXXPXXPXRXAKKK, where X represents all amino acids except cystein (Cys) and tryptophan (Trp), M stands for methionine (Met), A for alanine (Ala), R for arginine (Arg), P for proline (Pro) and K for lysine (Lys)) were used to determine the optimal binding motif of the SH3 domain of p85alpha and p85beta. The two amino acids Cys and Trp were excluded from the library in order to avoid problems due to oxidation of these residues during the Edman sequencing later. The first two amino acids were fixed to Met and Ala to verify proper sequencing and to allow quantification of the level of library present. Ala was fixed again at cycle 15 for quantification purposes: quantification of the peptide present and estimate how much peptide had been lost over the cycles before. The three lysines fixed at the C-terminus improved the solubility of the library. The theoretical degeneracy constitutes

18^9 potential peptide sequences. The library was a gift from Mike Yaffe (in brief: the synthesis was performed by Michael Berne at the Tufts Medical School according to the standard BOP/HOPt coupling protocol using a Peptide BioSynthesizer (ABI 431A). Poorly coupling amino acids were added in compensating amounts to achieve a theoretical probability of each amino acid of 1/18.). *(b) Binding of the degenerate peptide library to PI3K p85 SH3 domains and sequencing of the degenerate peptide mixture.* The GST-fusion proteins, preabsorbed on glutathione-sepharose beads, were incubated with 1mg peptide library in PBS (pH7.2), 1mg/ml BSA and 0.01% Nonidet P-40 for 1h at 4°C. The beads were washed three times quikly with PBS and then the peptides were eluted with 30% acetic acid. The peptide mixture was lyophilized, resuspended in distilled water and subjected to Edman Sequencing on an Applied Biosystems (Foster City, CA) Procise 494 Automated Protein Sequencer. The specificity for an amino acid in a particular position was determined by dividing the amount [pmoles] of a particular residue by the average amount of amino acids in that cycle (amino acid specific amount of a given position in a certain cycle / summe of all amino acid amounts in a given position in a certain cycle / 18). Afterwards, the data were corrected by dividing each value by the relative amount of the corresponding amino acid in the starting mixture. To control for non-specific binding of the library to the GST-beads, the screening and normalization was also performed with GST beads. The values obtained from screening the library with the GST-fusion proteins was then divided by the control values obtained from the screening with GST only.

4. Cell biological assays

Lamellipodia formation assay. Cells were seeded to various densities on glass coverslips in the morning and then starved overnight in DMEM. Like this the cells were not too adherent and still capable of detaching from the surface.

Time-lapse-Ruffling: The coverslips were placed into a heated device (37°C) on an inverted microscope. Basal ruffling was assessed for 3min and then various growth factors were added to stimulate the actin rearrangement processes. For example, PDGF-BB or PDGF-AA was added to a final concentration of 50ng/ml –100ng/ml, IGF-1 was used at 20nM and EGF was added to 100ng/ml. Every 15 seconds up to 120 pictures

were taken and movies were analyzed for the percentage of cells forming ruffles after growth factor stimulation.

Phalloidin staining of fixed ruffles: Cells on coverslips were prepared as just described above. The subconfluent, resting cells were stimulated with 50ng/ml PDGF-BB for 15min and then washed with CBS. The cells were fixed in CBS containing 3.7% paraformaldehyde for 20 min and then permeabilized for 10–20min in CBS with 0.1% triton X-100. The actin cytoskeleton was stained during the permeablilization process with rhodamine coupeled phalloidin (concentration). The coverslips were washed once in CBS and then mounted and analyzed under the microscope for the percentage of cells exhibiting lamellipodia formation.

Woundhealing assay. Confluent cells were starved over night. A wound was applied to the confluent monolayer with a yellow tip (Tip One) in form of a cross. The floating cells were washed off and DMEM with a final concentration of 10ng/ml PDGF, 20nM IGF-1, 100ng/ml EGF, 15%FCS +/- 10uM LY294002 was added. Pictures of the filling wounds were taken every 2h. In order to find the exact same position of the starting wound, always the southern part of the wounded cross was analyzed.

5. Physiological studies

Mice. Mice were maintained and all animal experiments were carried out according to institutional guidelines. Mice were housed in a barrier facility in microisolator cages. Mice heterozygous for all p85alpha gene products (p85a+/-p55a+/-p50a+/-) were crossed to generate p85a-/-p55a-/-p50a-/- embryos or crossed with mice lacking p85beta to obtain double heterozygous mice. These double heterozygous mice (p85a/p55a/p50a/p85b+/-) were crossed with p85beta-/- mice to generate p85a+/-p55a+/-p50a+/-p85b-/- mice, which were viable and fertile. Intercrosses of these mice were performed to generate embryos.

Mice homozygous for p85alpha (p85a-/-), lacking only the p85alpha isoform but retaining p55alpha and p50alpha, were purchased from Taconic Farms. These animals, in the C57Bl/6 background, were crossed with p85beta null mice (129SvEv x C57Bl/6) under a research crossbreading license. The resulting double heterozygous mice were

crossed with p85beta null mice to generate p85a+/-p85b-/- mice, which were viable and fertile. Intercrosses of these mice were performed to generate embryos.

Mice on a 129 background with heterozygous loss of p110a (p110a+/-) or p1110b (+/-) were intercrossed to produce wild-type, single heterozygous and double heterozygous mice. All animals were fed a standard rodent chow (Purina) or put on a high fat diet (Teklad) after they reached 2 months of age. All study groups contained littermates of the same gender that were born within the same week.

Analysis of embryos. Timed pregnancies of experienced breeding pairs of either p85a+/-p55a+/-p50a+/-p85b-/- mice or p85a+/-p85b-/- mice were analyzed. Therefore, the females were checked between 8-11am for the presence of a sperma plug. In case a plug was observed, that morning was counted as embryonic day E0.5. Potentially pregnant females were sacrificed and dissected between E8-13.5. The embryos were removed and placed in PBS. In case the embryos were < E9, FCS was added to the PBS to about 10%. The yolk sac was removed and used for genotyping by PCR (previously described by Fruman et al. and Terauchi et al.). The embryos were either photographed or placed in fixative (Bouin's solution or formalin) for further analysis.

Histopathology of embryos lacking all gene products of p85alpha and p85beta. The specimens were fixed in Bouin`s solution for 3-4h and then transferred into 70% ethanol for a variable amount of time (depending on the specimen size). I performed the dehydration process myself according to the guidelines described in (The Atlas of Mouse Development, Kaufman, Academic Press) in order to be able to properly orient the E8-9 old embryos in the paraffin block. Sagittal sections were stained by standard haematoxylin and eosin (H&E) staining procedure or a TUNEL assay was performed according to the manufacturer's protocol (In situ cell death detection kit TMR red, Roche).

TUNEL STAIN of whole mount embryos lacking all gene products of p85alpha and p85beta. E8 old embryos were fixed for 3h in formalin. In order to protect the embryo from physical damage and to guarantee fast transfer from the mmediat to the fixative the yolk sac was not removed prior to fixation. The whole mount specimens were analyzed for apoptosis activity using a TUNEL assay kit (In situ cell detection kit POD, Roche).

Metabolic Studies. All blood samples were obtained by tail bleeding. For assessment of plasma insulin or blood glucose concentrations, the blood samples were collected at 11pm (fed levels) or the mice were starved over night and the samples were collected at 9am (fasted levels) the next morning. For plasma insulin concentrations, the tail blood was collected into heparinized microcapillaries (about 200ul). The samples were spun for 10min at 5000rpm and the insulin concentration in the supernatant was determined by ELISA. Blood glucose levels were determined from whole venous blood by using an automatic glucose monitor.

For the **glucose tolerance test (GTT)**, blood samples were analyzed every at 0, 15, 30, 60, 120 and 180 min after injection of 2 g/kg glucose in PBS. Therefore, overnight-starved mice are injected intraperitoneally with glucose normalized to their body weight. Then, the uptake of blood glucose into peripheral organs (muscle, fat and to a lesser extent into liver) is assessed by its clearance from the blood. Abnormalities in this test might result (a) from defective insulin production by the pancreas and/or (b) from impaired/improved insulin signaling in the peripheral tissues. In case a mouse model has a slower glucose uptake than wild-type mice, they are considered as glucose intolerant. Is the drop of glucose levels similar or faster to wild-type mice, they are glucose tolerant.

For the **insulin tolerance test (ITT)**, blood samples were analyzed at 0, 15, 30, 45, 60 and 90 min after injection of 0.5 – 1.5 units/kg regular human insulin, dependent on age and gender of the animals. (In detail: female mice were injected with 0.5U/kg at 2months of age, 0.75U/kg at 4months of age and 1U/kg at 6months of age. Male mice where injected with 0.75U/kg at 2months of age, 1U/kg at 4months of age and 1.5U/kg at 6months of age.) ITTs analyze the ability of a mouse to clear basal blood glucose upon insulin injection. This test bypasses pancreatic function, assessing directly the ability of the peripheral organs, such as muscle, fat and to a lesser extent liver to take up glucose. If

the glucose drop is faster than the wild-type response the mouse model is considered as hypersensitive to insulin, in the opposite case it is insulin resistant.

Preparation of liver and muscle lysates from unstimulated and insulin-treated mice.

Four months old female mice were starved over night and anesthetized with pentobarbital for about 5min or until they were not reacting to pinching of their legs. Then, they were injected with 5 units of regular human insulin or PBS into the inferior vena cava. After 5min, first the liver and then the quadracepts muscles were removed and immediately frozen in liquid nitrogen. The frozen tissues were homogenated with a tissuemizer in buffer A. The lysates were spun at 3,000rpm for 3min at 4°C and the supernatant without the upper, fatty layer was subjected to a highspeed spin in a Ti70.1 rotor at 55,000rpm for 1h at 4°C. The supernatant avoiding again the upper, fatty layer was transferred to a conical tube and the protein concentration was determined via the Bradford assay. Half a liver yielded in approximately 8mg total protein, whereas a muscle provided about 4mg total protein.