CHAPTER 2

MATERIALS AND METHODS
MATERIALS AND METHODS

2.1. Materials

2.1.1. Sources of Chemicals

Acrylamide, bis-acrylamide, Agarose, β-mercaptoethanol, BSA fraction V, BCIP, calcium chloride, cisplatin, cycloheximide, cytochalasin D, DMSO, DTT, EDTA, ethidium bromide, fibronectin, HEPES, glutathione agarose beads, glutathione (reduced), IPTG, kanamycin, leupeptin, lysozyme, NBT, penicillin, PIPES, PMSF, Ponceau S, sodium bicarbonate, sodium dodecyl sulphate (SDS), sodium ortho-vanadate, staurosporine, streptomycin, TEMED, Tris base, Triton X-100, trypsin, Tween-20, X-gal were all obtained from Sigma. Medium molecular weight marker proteins for SDS-PAGE, DNA markers and PCR reagents were from Bangalore Genei & New England Biolabs. Lipofectamine 2000, Lipofectamine-PLUS reagent, FBS and DMEM were obtained from Invitrogen. Jasplakinolide, Nocodazole, RNase A and Glycerol were from Calbiochem. Hybond C, Hybond-ECL membranes were purchased from Amersham Life Science. Western lighting chemiluminescence reagent was from Perkin Elmer Life Science. Protein A/G PLUS agarose beads were from Santa Cruz. Complete (with EDTA) and Incomplete (without EDTA) protease inhibitors were from Roche. Columns for preparing transfection grade DNA were from Qiagen or Life Technologies. Restriction enzymes, T4 DNA ligase were from New England Biolabs and Bangalore Genei. Tryptone, agar and yeast extract were from Difco Laboratory and Blotto was from Santacruz. Whatman filter papers were from Whatman International Ltd; X-ray films were from Konica Corporation or Kodak. Intensifying screens were obtained from Dupont. Phosphor-imager screens were from Fuji. All ³²P-radiolabelled nucleotides were obtained from BRIT, Mumbai. STI 571 was a gift from Natco Pharma Ltd. Oregon green phalloidin and Rhodamine phalloidin used to detect F-actin were from Molecular Probes. In Situ Cell Death Detection Kit, TMR red, for TUNEL assay was from Roche. Z-VAD-
FMK was from BD Pharmingen. Other reagents were from local suppliers like Himedia, Qualigens, SRL, SD fine-chemicals Ltd., and Merck India Ltd. and were of analytical grade.

2.1.2. Expression constructs

1. pCDNA3.1(+) : This mammalian expression vector (5.4 kb in size) was obtained from Invitrogen Life Technologies.
2. pEGFP (C2/C3) : These mammalian expression vectors were obtained from Clontech Laboratories Inc.
3. pGEX-5X2 : This bacterial expression vector for making GST-fusion protein constructs, was obtained from GE Healthcare formerly Amersham Biosciences.
4. The human c-Abl expression vector c-Abl pSGT, the constitutively active c-Abl mutant (ΔXB – with deleted SH3 domain) and the catalytically inactive c-Abl construct K290M/ KD-Abl were kindly provided by Dr. Richard Van Etten (Tuft University).
5. Full-length human C3G cloned in pCDNA3-FLAG was kindly provided by Dr. S. Tanaka (Hokkaido University School of Medicine).
6. Y504F mutant of C3G in which tyrosine 504 is mutated to phenylalanine were kindly provided by Dr. M. Matsuda, Department of Pathology, Research Institute, International Medical Centre of Japan, Tokyo, and have been described by Ichiba et al.
7. The plasmid expressing the Crk-binding region (CBR) was kindly provided by Dr. P.J.S. Stork (Oregon Health & Science University).
8. GFP-RalGDS-RBD construct was kindly provided by Dr. P.J.S. Stork (Oregon Health & Science University).
9. GFP-Rap1GAP construct was from Dr. Patrick Casey (Duke University).
10. Wild type human Hck cDNA cloned into pCDNA6 expression vector was a kind gift of Dr Todd Miller (State University of New York, Stony Brook).
11. CrkII and W170K mutant were gifted by Dr. Bruce Mayer (University of Connecticut Health Center).
12. Clone C-9 of rat C3G has been described previously (Shivakrupa et al., 2003). Clone C-9 and its fragments were cloned in the EcoRI site or
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BamHI site of pGEX vector to produce GST fusion proteins.

13. The GST-CBR construct was generated by sub-cloning human CBR fragment into pGEX-5X2 vector at BamHI and SalI sites.

14. c-Abl with deletion of its actin binding domain (ΔFABD) was generated by inserting a stop codon immediately after amino acid 1119 at C-terminal region of c-Abl-pSGT construct using a PCR-based site-directed mutagenesis approach. The deletion construct ΔFABD devoid of the C-terminal 30 amino acids (1120-1149) lacks ability to bind F-actin as reported earlier (Woodring, 2001).

15. Vectors for shRNA expression used in this study to target human C3G (ShC and mutant ShC - mShC) (as described in Radha et al., 2007) had been constructed using the U6 promoter-based system. ShC targeted the C3G sequences: 5'-GGATGCTGAGCTCTTCTATAA-3', i.e., nucleotides 2766-2786 accession number, NM-198679. The desired synthetic oligos had been annealed and cloned in the BbsI-XbaI digested mU6-pro plasmid. Oligonucleotides with two residues mutated had been used for construction of mutant shRNA constructs.

16. GFP-p23 plasmid was gifted by Dr Irene Schulz (University of Saarland, Germany).

2.1.3 Antibodies

Antibodies used to carry out the experiments are listed below:

1. Rabbit polyclonal and mouse monoclonal antibodies against C3G used for indirect immunofluorescence and immunoblotting were from Santa Cruz.

2. Polyclonal antibody (C9) raised in our laboratory which detects overexpressed constructs of C3G specifically, was used for detection of C3G and CBR constructs in indirect immunofluorescence.

3. The rabbit polyclonal anti-pY504-C3G (SC-12926 R), which recognizes human C3G phosphorylated on tyrosine 504 was from Santa Cruz.

4. c-Abl (mouse monoclonal)

5. PY20 (mouse monoclonal)

6. α-tubulin (mouse monoclonal)

7. Hck (rabbit polyclonal)
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8. pH2B(S14) (rabbit polyclonal) and
9. Cdk-2 antibodies were purchased from Santa Cruz Biotechnology
10. Control antibodies for immunoprecipitation: anti-mouse monoclonal IgG and anti-rabbit polyclonal IgG were from Santa Cruz
11. Secondary antibodies for western blotting and immunofluorescence were from Bangalore Genei, Amersham LifeScience or Vector labs.
12. Phospho-c-Abl (anti-pY245)
13. Phospho-c-Abl (anti-pY412) and
14. phospho-CrkII (anti-pY221) antibodies were from Cell Signaling Technology.
15. Cleaved caspase-3 antibodies were from Cell Signaling Technology
16. γH2AX antibody, mouse monoclonal, was from Upstate Biotechnology
17. Giantin antibody, mouse monoclonal, was from Abcam

2.1.4 Bacterial Strains
1. E. coli DH5α: F' end A1 hsd R17 (rK-, Mk+) sup E44 thi-1 rec A1 gyr A96 (Nal') rel A1 Δ (lac ZYA-arg F) u169 (φ80 lac Z8 M15): This strain of Escherichia coli was used for all transformations, plasmid isolations and for selection of recombinant clones.
2. E. coli BL21 (DE3): F- ompT gal dcm lon hsdSB(rB- mB-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]): This strain of Escherichia coli was used for expression and purification of recombinant GST-fusion proteins.

2.1.5 Cell Lines
The cell lines used in this study and their tissue types are listed below.
1. Cos-1 - African green monkey (Cercopithecus aethiops) kidney cell line transformed by SV 40, established from CV-1 Simian cells
2. MCF-7 - human breast carcinoma
3. HEK 293 - human embryonic kidney
4. HeLa - human cervical adenocarcinoma
2.1.6 Bacterial Media, Antibiotics and Chemical Stocks

**Acrylamide solution (30%):** 29.2 g of acrylamide and 0.8 g of N, N'-Methylene bis-acrylamide was dissolved in double distilled water, made up to 100 ml and stored at 4°C in an amber-coloured bottle.

**Agarose gel loading buffer (6X):** 0.25% bromophenol blue and 0.25% xylene cyanol in 30% glycerol.

**Amido-black staining solution:** 0.1% amidoblack 10B, 25% isopropanol and 10% acetic acid in water.

**Ampicillin:** 1000X stock solution was made by dissolving 100 mg Ampicillin per ml of sterile double distilled water.

**BCIP:** 50 mg/ml stock solution was made using di-sodium salt of BCIP in double-distilled water and stored at -20°C.

**Buffer saturated phenol:** Melted phenol was mixed with equal volume of 0.5M Tris-HCl pH 8.0 and 0.1% 8-hydroxyquinoline and mixed well. The upper aqueous phase was removed and the process repeated with 0.1M Tris-HCl pH 8.0. This step was repeated till the pH of the aqueous solution was equilibrated to 8.0. It was mixed with 0.1 volume of 0.1M Tris-HCl (pH 8.0) and stored in amber-colored bottle at 4°C.

**Chloroform:Isoamyl alcohol:** 24:1 (v/v) chloroform:isoamyl alcohol mixture was prepared.

**DMEM (1X):** 13.4 g of DMEM and 3.7 g of NaHCO₃ was dissolved in milli-Q water to make 1 litre of the medium. The pH was adjusted to 7.4 using HCl and subsequently filter sterilized.

**DTT (1M):** 3.09 g of DTT was dissolved in 20 ml of 0.01 M Sodium acetate (pH 5.2), sterilized by filtration and stored as aliquots in -20°C.

**Ethidium Bromide:** 10 mg/ml solution in water.

**LB Agar:** LB media reconstituted with 1.5% Bactoagar was used for pouring LB plates.

**Luria-Bertani (LB) broth:** 1% Bactotryptone, 1% NaCl and 0.5% Bacto-yeast extract were dissolved in double distilled water. pH was adjusted to 7.4 using 10N NaOH and then autoclaved.
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Mountant: 90% glycerol, 10% antifade solution (10 mg/ml para-phenylene diamine hydrochloride in 10X PBS) and 0.5μg/ml DAPI (from 5 mg/ml stock) in 1X PBS.

NBT: 50 mg/ml stock solution was made in 70% Dimethyl formamide and stored at -20°C.

PBS (1X): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.4 mM KH₂PO₄. A 10X stock solution was prepared in double distilled water, pH adjusted to 7.4 with HCl and autoclaved.

Penicillin, streptomycin, kanamycin: 600 mg penicillin, 1000 mg kanamycin, 500 mg streptomycin were dissolved in 100 ml 1X PBS and filter sterilized to make a 100X stock which was stored frozen at -20°C. 1X antibiotic solution was used in all tissue culture media.

Ponceau S: A 10X stock of Ponceau S was made containing 2% Ponceau S, 30% trichloroacetic acid and 30% sulfosalisylic acid in water. For use with PVDF membranes, 1X solution of 0.1% Ponceau S in 5% acetic acid solution was used.

RNase A: 20 mg/ml stock solution was made in 15 mM NaCl and 10mM Tris (pH 7.5). It was then boiled for 15 minutes and cooled slowly to room temperature. The stock was then aliquoted and stored at -20°C.

SDS-PAGE sample buffer (3X): 180mM Tris-Cl pH 6.8, 6% SDS, 15% glycerol, 7.5% β-mercaptoethanol and 0.01% bromophenol blue in double-distilled water and stored at -20°C.

TBE (1X): 89 mM Tris base, 89 mM boric acid and 1 mM EDTA dissolved in double distilled water. 10X stock solution prepared was filtered, autoclaved and stored at room temperature.

Tris EDTA pH 8.0 (TE): 10 mM Tris HCl (pH 8.0) and 1 mM EDTA (pH 8.0).

Trypsin EDTA: 0.125% trypsin (cell culture grade) and 0.1% EDTA was dissolved in 1X PBS, filter sterilized and stored at -20°C.

2.2 Methods

2.2.1 Sterilization

All glassware and plastic ware were sterilized by autoclaving at a steam pressure of 15 psi at 121°C for 20 min. Sterile, disposable material was used for
cell culture techniques. Solutions were prepared in double-distilled water and generally sterilized by autoclaving or filter sterilized through a sterile 0.45 μm nitrocellulose filter (Millipore). Bacterial growth media were autoclaved for 40 min at 15 psi at 121°C. Cell culture media, antibiotics and trypsin were sterilized by filtration through 0.2 μm filters.

2.2.2. Plasmid Isolation

Plasmid DNA miniprep was carried out by alkali lysis method as described by Sambrook et al., 1989 with certain modifications. Restriction digestion of plasmids for screening purposes was carried out after the Rnase treatment. In order to prepare plasmids for sequencing purposes, an alternate method of plasmid purification from technical manual of the ABI prism 3700 sequencer was used. In this method, 3 ml of overnight grown bacterial culture was pelleted and re-suspended in 100 μl of double-distilled water. 100 μl of lysis buffer (100 μl of 10% SDS, 20μl of 0.5M EDTA and 10 μl 10N NaOH made up to 1ml with water) was added and the tube was kept in boiling water for 2 minutes. 50 μl of 1M MgCl₂ was added to these, mixed and kept on ice for 2 minutes. The mixture was spun at maximum speed in a microcentrifuge for 2 minutes. 50 μl of 5M potassium acetate was added to the supernatant and kept on ice for 2 minutes. The tube was then spun at maximum speed for 2 minutes and the supernatant transferred to a separate tube. DNA was precipitated by adding 600 μl of Isopropanol followed by 2 minutes incubation on ice. The tube was then spun at maximum speed for 2 minutes to pellet the plasmid DNA. The pellet was washed with 1 ml of 70% ethanol and air-dried. The pellet was subsequently re-suspended in 100μl TE pH 8.0 containing RNase A.

Plasmids for transfections were prepared using QIAGEN-tip20 (miniprep) and QIAGEN-tip 100 (midiprep) columns according to manufacturer's instructions. The protocol involves alkaline lysis of cells followed by column purification of DNA that yields high purity plasmids with relatively low levels of impurities.

2.2.3. Quantitation of Nucleic Acids

The nucleic acid concentration was determined by measuring the absorbance at 260 nm (Maniatis et al., 1982). Empirical relationship of 50 μg of
double stranded DNA, 33 μg of single stranded DNA or 40 μg of single stranded RNA was taken to be equal to 1.0 OD$_{260}$. Purity of the preparation was estimated using ratio of absorbance at 260 nm to 280 nm.

2.2.4. Agarose Gel Electrophoresis

DNA and RNA samples were mixed with 6X loading dye (so as to make it 1X) and were resolved, using 0.8-1.2% agarose gels made in 1X TBE buffer. 0.25 μg/ml ethidium bromide was added to gels during preparation for visualizing DNA.

2.2.5. Restriction Endonuclease Digestion

Plasmid DNA (1-2.5 μg) was digested with 1-2.5 units of restriction enzyme in a compatible buffer in a 20 μl final volume, as per manufacturers instructions. Digested products were visualized by resolving in an agarose gel along with appropriate DNA markers.

2.2.6. Gel Elution of DNA Fragments

The GeneClean purification kit from Bio101 was used to purify DNA by gel elution. Eluted DNA was used for further protocols such as ligation.

2.2.7. Ligation

DNA fragments obtained after gel purification of PCR products or restriction digests were ligated using T4 DNA ligase at 22°C (for blunt ended cloning) or 16°C for 8 hours to overnight. A molar ratio of vector to insert of 1:3 was generally used.

2.2.8. Preparation of Ultracompetent Cells

The method of Inoue et al., 1990 was used for high efficiency ultracompetent cells. Pre-inoculum of E.coli DH-5α or BL-21 strains from a single colony was grown overnight in 10 ml of LB at 37°C. 0.1% inoculum of this culture was added to 250 ml LB medium in a 2-litre flask and kept under vigorous shaking at 18°C till absorbance reached about 0.6. The culture was kept on ice for 10 min and the cells harvested by centrifuging at 2500 g for 10 min at 4°C. The cell pellet was resuspended in 80 ml of ice-cold filter sterilized PIPES buffer.
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(10 mM PIPES, 15 mM CaCl₂, 250 mM KCl, 55 mM MnCl₂ pH 6.7) and placed on ice for 10 min. Cells were re-centrifuged and gently resuspended in 10 ml of PIPES buffer with 7% DMSO. The cells were kept on ice for about 10 min, aliquoted and snap frozen using liquid nitrogen for storage at -80°C.

2.2.9. Transformation of *E. coli*

Ultracompetent cells (50 μl) were thawed on ice, 5 μl of the ligated mixture or 5-10 ng of pure plasmid was gently mixed with the cells and incubated on ice for 30 minutes. Heat shock was given at 42°C for 90 seconds and immediately kept on ice for 1-2 minutes. 200 μl of LB was added for recovery and the tube was incubated at 37°C for 45 minutes. Cells were then spread on LBA plates containing proper antibiotic and plates were incubated overnight at 37°C for colonies to grow.

2.2.10. DNA Sequencing

ABI Prism Model 3700 DNA Analyzer or Model 3730 DNA Analyzer were used for all DNA sequencing reactions. 200 ng of plasmid DNA and 2.5-5 pmoles of primer was constituted in a volume of 3.2 μl and mixed with 1.8 μl of the big-dye terminator sequencing kit (Perkin-Elmer). PCR was carried out in the GeneAmp PCR System 9600 Thermal Cycler with denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec and extension at 50°C for 4 min, for a total of 35 cycles and a final extension was done for 5 minutes. DNA was ethanol precipitated and washed with 70% ethanol. The samples were then resuspended in 10 μl High-dye-formamide (Perkin-Elmer) and 5 μl of this was loaded in the capillaries.

2.2.11. Polymerase Chain Reaction (PCR)

PCR was done in a reaction mix containing 1X PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin- Taq buffer 10A from Bangalore Genei), 250 mM each of the dNTPs, 200 ng primers and 1.5 units of Taq DNA polymerase. After an initial denaturation at 94°C for 2 min, required number of cycles was carried out. Each cycle consisted of denaturation at 94°C
for 30 sec, annealing at 4°C below melting temperature of the primer for 1 min and extension at 72°C for 1 min. A final extension for 7 min was given at 72°C for completion of elongation products.

2.2.12. Construction of GST fusion proteins of CBR (Crk binding region of C3G)

To construct the GST fusion protein of CBR, the Crk binding region of C3G, the region consisting of 960-2099 nucleotides of human C3G cDNA was amplified by PCR. The PCR product was checked for amplification and subsequently the PCR fragment was excised from gel and gene cleaned using PerfectPrep Gel Clean Up Kit (Eppendorf). This purified PCR fragment was restriction digested with BamHI and SalI enzymes at 37°C for 4 hours and then ligated with already digested (BamHI and SalI) pGEX-5X2 vector in the ratio of 1:5 overnight at 16°C. 5 ml of ligated product (reactions with and without the insert) was transformed into ultra competent E. coli DH5α cells, and colonies were allowed to grow overnight at 37°C. The colonies, which grew only on those plates where ligation mix had insert, were further screened by PCR to confirm the presence of insert and given for DNA sequencing. The primer used for construction of GST fusion proteins is listed in Table 2.1:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>YC3F</td>
<td>5'-CGGGATCCGGATGTCACAGTCAACTGAG-3'</td>
<td>Forward primer with BamHI end (underlined)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55°C; 1139 bp</td>
</tr>
<tr>
<td>YC3R</td>
<td>5'-CGCGTCGACGGATGTCCTTCTTCCCAG-3'</td>
<td>Reverse primer with SalI end (underlined)</td>
</tr>
</tbody>
</table>

2.2.13. Construction of ΔFABD of c-Abl by site-directed mutagenesis

Mutagenic primers containing the desired nucleotide changes were purified by urea-PAGE and mutations were generated by PCR using Quik change site directed mutagenesis (Stratagene) protocol. The primers were generally 30 bp and designed to have mutations in the middle of the primer sequence. The primers had a minimum 40% GC content and the Tm of the primers was greater
than or equal to 78°C. The $T_m$ was calculated by the formula: $81.5 + 0.41(\%GC) - 675/N - \%$ mismatch, where $N$ is the primer length in base pairs. Typically, 25$\mu$l PCR reaction was set up using 10-20ng of DNA template, 125ng of the mutagenic primers, 200$\mu$M of dNTPs, 2.5$\mu$l of 10X $Pfu$ buffer and 1.25 units of $Pfu$ Turbo DNA polymerise which has proof reading activity. The cycling conditions were: initial denaturation at 95°C for 2 minutes, followed by 16-18 cycles of 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 68°C for required time (calculated according to 2 minutes extension per Kb of plasmid). The final extension was given at 72°C for 7 minutes. The PCR product (5$\mu$l) was run on 0.8% gel to confirm amplification. The PCR product (10$\mu$l) was digested with DpN I enzyme (2.5 units) at 37°C for 4 hours. 5$\mu$l of digested product was then transformed using ultra competent DH5-α cells and colonies were allowed to grow overnight in a 37°C incubator. Since DpN I digests the methylated DNA, all parental plasmid gets digested leaving the newly synthesized unmethylated DNA strand. The control parental plasmid of equal concentration was also digested with DpN I and then transformed to assess the efficiency of digestion as in principle no colonies must grow on plate transformed with parental plasmid after digestion. These colonies were picked and plasmids made which were checked by PCR using check primers (these primers are designed in a way that they end at 3' mutated nucleotide), which will amplify only that cDNAs which have mutation in it and not the unmutated cDNA.

The list of all the mutagenic primers and corresponding check primers is given below in table 2.2.

**Table 2.2: List of primers used for site directed mutagenesis**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>5'-GGAGCTTCAGATCTGACCGGCGACAGG-3'</td>
<td>55°C; 16 cycles; TGC (Cys) to TGA (Stop)</td>
</tr>
<tr>
<td>P2</td>
<td>5'-CCTGCTGTCGCCGGTCAGATCTGAAGCTCC-3'</td>
<td>At 1119 amino acid position Deletion of 31 amino acids at C-terminal</td>
</tr>
<tr>
<td>ChF</td>
<td>5'-CATCCCTCTCATATCAACCG-3'</td>
<td>Check primer, 61°C,</td>
</tr>
<tr>
<td>ChR</td>
<td>5'-CTGCCTGCTGTGCGCCGGT-3'</td>
<td>311 bp</td>
</tr>
</tbody>
</table>
2.2.14. Mammalian Cell Culture

Cell lines were maintained in DMEM supplemented with 10% FBS with penicillin, streptomycin and kanamycin at 37°C in a humidified 5% CO₂ containing incubator. Sub-culturing was carried out by incubating the cells with trypsin-EDTA solution after rinsing with sterile 1X PBS.

2.2.15. Transfection of DNA in Mammalian Cells

For Cos-1 cells, transfections were performed on cells grown as a monolayer in either 18 mm dish, 60 mm dish or coverslips using the cationic lipid DHDEAB as described (Radha et al., 2004). Briefly, 1 µl lipid diluted in 50 µl serum free DMEM was mixed with 1 µg DNA in 50 µl serum free DMEM. The mixture was kept at room temperature for 30 minutes to allow complex formation before adding to the (60%-80% confluent) cell monolayer. Transfections were stopped after 4 hours by adding complete DMEM to the cells and then harvested at indicated time points after transfection.

HEK293, MCF-7 and Hela cells were transfected with required plasmids using Lipofectamine 2000 reagent according to the manufacturer's instructions. Cells were trypsinized and required number plated in antibiotic free medium the day before transfection. Plasmids (Total of 500 ng per cover slip, 500 ng per 18-mm well and 4 mg for each 60 mm dish) and Lipofectamine 2000 (1 µl for coverslips and 4-5 µl for 60 mm dishes) were diluted in serum-free DMEM, mixed, incubated for 20 minutes and added on to the cells. Transfections were stopped after 5 hours by replacing transfection medium with complete DMEM.

When C3G or c-Abl was co-transfected with other constructs, amounts of DNA used were maintained by using empty vectors as controls.

2.2.16. Co-immunoprecipitation

For co-immunoprecipitation, untransfected Cos-1 cells, or those transfected with c-Abl and C3G were lysed in IP buffer containing 20 mM Tris 7.4, 1% Triton, 5mM EDTA, 0.1% BSA, 150mMNaCl, 1mM PMSF and protease inhibitor cocktail from Roche. Clarified lysates were incubated with equal amounts of c-Abl antibody or control mouse IgG overnight. The complexes were pulled down using protein A/G plus agarose beads (Santa Cruz) and washed with wash buffer (50mM Tris-Cl pH 7.5, 150 mM NaCl, 10% Glycerol, 0.1% Troton-X-)
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100). The proteins were eluted by boiling the bead complexes in 3X SDS sample buffer, which were then subjected to SDS PAGE and Western blotting using anti-C3G and anti c-Abl antibodies.

2.2.17. Expression and purification of recombinant proteins

Expression of recombinant proteins was carried as described earlier (Radha et al., 2007). GST (glutathione-S-transferase) and GST-C3G-CBR fusion protein were expressed in Escherichia coli BL-21 strain and protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37 °C. Cells were harvested and the pellet was resuspended in 1 ml of PBS containing 1 mM PMSF and protease inhibitors. Cells were sonicated with bursts of 5 s with cooling on ice for 5 s. To solubilize the proteins, 1% Triton X-100 was added and left on ice for 20 min. The clarified supernatant was incubated with preswollen glutathione sepharose beads (50% slurry in PBS) for 1 h at 4 °C. The beads were pelleted, washed and stored in PBS containing protease inhibitors and 10% glycerol at 4 °C, to be used for pull down assays.

For elution of the protein from the beads, the beads were incubated with 5mM glutathione made in 50mM Tris-HCl pH 9.0 for 1-2 min. The beads are spun down and the eluted protein present in the supernatant was used for the in vitro kinase assay.

2.2.18. GST-pull down assay

Cos-1 cells were transfected with c-Abl or CrkII expression plasmids and lysed in buffer containing 10mM Tris pH 7.4, 150mM NaCl, 5mM EDTA, 1mM PMSF, 1% Triton X-100, 0.1% BSA, 2 mM Na₃VO₄, 10 mM NaF, and protease inhibitors. The clarified supernatant was incubated with GST fusion proteins bound to beads for 6 h at 4 °C. The bead complexes were then pelleted and washed thrice with wash buffer containing 20 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100 and protease inhibitors. The bound proteins were eluted by boiling the bead complexes in 3X SDS sample buffer which were then subjected to Western blotting.
2.2.19. **In-vitro phosphorylation assay**

c-Abl was immunoprecipitated from lysates (made in 2X IP buffer – 40mM Tris pH 7.2, 2% Triton-X-100, 300mM NaCl, 5mM EDTA, 2mM PMSF, IX protease inhibitor; then diluted with cold H2O) of Cos-1 cells expressing WT c-Abl or K290M constructs. The beads containing immune-complexes were washed with wash buffer, followed by final wash in 2X kinase buffer (25mM Tris pH 7.5, 10mM MgCl2, 10 mM MnCl2, 1mM DTT, 1 mM PMSF, 100 μM Na3VO4, 2μM ATP). The complexes were then mixed with purified GST or GST-CBR (2-4 μg) and kinase reaction carried out in kinase buffer (in 25μl reaction volume) using 30 mCi of γ32P-ATP for 30 minutes at 37°C. The reaction was terminated by addition of 3X-SDS sample buffer followed by boiling for 5 minutes. The reaction components were analyzed by SDS-PAGE. The gel was stained with coomassie blue to visualize expression of proteins and subsequently dried for autoradiography or phosphor image analysis.

2.2.20. **SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

SDS-PAGE, as described by Laemmli, 1970, was carried out using a discontinuous buffer system. Stacking gel (0.125 M Tris-HCl pH 6.8, 5% acrylamide and 0.1% SDS) and the resolving gel (0.375 M Tris HCl pH 8.8; 8,10 or 12 % acrylamide and 0.1% SDS) were polymerised using TEMED and Ammonium per sulphate. The gels were run using buffer containing 0.025 M Tris, 0.192 M glycine and 0.1% SDS at 20 mA constant current. After stacking of proteins at the resolving front and subsequent entry into the resolving gel, current was increased to 30 mA till the end of the run.

2.2.21. **Western Blotting**

Proteins resolved using the SDS-PAGE gel were blotted onto nitrocellulose membranes (Hybond C from Amersham or Immobilon P from Millipore) using the semidry apparatus (Pharmacia). The semidry transfer buffer containing 39 mM glycine, 48 mM Tris-HCl, 0.0375% SDS and 20% methanol was used and transfer was carried out at constant current at 1 mA/cm² for 1-2 hours. After transfer, the proteins were stained using Ponceau S solution and the
positions of molecular weight markers were marked using pencil. Subsequently, blots were incubated in 5% Blotto (Santacruz) in TBST (10 mM Tris HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) for 1 hour at room temperature. The blots were then incubated with the required dilution of primary antibody in 0.5% Blotto in TBST for 1-2 hours at room temperature or overnight at 4°C. Non-specifically bound primary antibody was removed by three 5-minute washes with TBST. Subsequently, blots were incubated with appropriate dilution of secondary antibody in 0.5% Blotto in TBST for 60 minutes. After three washes with TBST, blots were processed for alkaline phosphatase reaction or enhanced chemiluminescence (ECL) detection. For alkaline phosphatase colour reaction the blot was developed with 0.1 M Tris HCl pH 9.0, containing 10 mM each MgCl₂ and NaCl, 0.005% BCIP and 0.01% NBT. ECL was done using the Western lighting chemiluminescence reagent from Perkin Elmer and different exposure times were given to get the right intensity of the signal. The membranes were stripped prior to incubation with other antibodies.

2.2.22. Treatment of cells

Cells were subjected to pervanadate treatment (as described earlier by Radha et al., 2004). by the addition of a freshly prepared 50μM solution of pervanadate for 10 minutes prior to harvesting. Pervanadate stock solution (50 mM) was prepared by mixing equal volumes of 100 mM solution of H₂O₂, with 100 mM solution of sodium orthovanadate. It was added to the cells within 5 mins of preparation.

To disrupt actin cytoskeleton, cells were treated with 1 μg/ml cytochalasin D for 20 mins. For stabilization of the actin cytoskeleton the cells were treated with 200 nM Jasplakinolide (Calbiochem) for 20 mins before fixation. Microtubule cytoskeleton disruption was performed by treating cells with 5 μg/ml of nocodazole (Calbiochem) for 30 mins prior to fixation.

For detergent extraction, coverslips with Cos-1 cells expressing the desired constructs were given a quick wash with PBS containing 0.1 mM MgCl₂ and 0.1 mM of CaCl₂ followed by treatment with 0.5% Triton-X-100 in PBS for 12 seconds prior to fixation. Duplicate coverslips were kept untreated and fixed.
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along with the treated ones, followed by subjecting them to immuno-staining with required antibodies and phalloidin.

To induce apoptosis Cos-1 cells transfected with GFP or c-Abl were either left untreated (Utr) or treated with Staurosporine, STS (0.3 μM, 3.5 hours) prior to fixing. Inhibition of apoptosis was done using a pan-caspase inhibitor, ZVAD-FMK (20 μM, 24 hours).

2.2.23.1 Immunofluorescence and confocal microscopy

For immunofluorescence, the cells were grown on cover slips and processed essentially as described by Radha et al., 2004. The secondary antibodies used were rabbit or mouse IgG coupled with either Cy3 (Amersham), Alexa 488 or Alexa 633 (Molecular Probes) fluorescent dyes. F-actin was detected using phalloidin conjugated to either Rhodamine or Oregon green dyes.

The cells, after required treatments or transfections, were washed with PBS and fixed with 3.7% formaldehyde in 1X PBS for 10 min at room temperature. They were then permeabilized using 0.5% Triton-X 100 and 0.05% Tween-20 in 1X PBS for 6 minutes at room temperature. The cells were then washed and incubated with PBS containing 2% BSA for 1 hour at room temperature for blocking. They were then incubated for required time with primary antibody that is diluted in PBS containing 2% BSA. After washing with PBS, cells were incubated with fluorophore-conjugated secondary antibody in blocking solution for 1 hour at room temperature. The cells were again washed with PBS and mounted on glass slides in mountant.

Immunofluorescence staining was observed and digital images were captured either with the LSM 510 Meta Confocal Microscope (Carl Zeiss), Leica TCS SP5 confocal microscope (Leica Microsystems) or with a Zeiss Axioplan2 microscope fitted with an Apotome. The confocal images were captured using a 20X/0.7 NA dry or 63X/1.4 NA oil immersion objectives. The images from Axioplan2 microscope were captured using a 40X/0.75 NA dry objective. Similar parameters of image capture were used for analysis of coverslips belonging to a particular experiment. Imaris software package (BitPlane, Switzerland) was used to reconstruct 3-D images from the stacks of confocal images.
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2.2.24. Estimation of apoptosis and TUNEL assay

Quantitative analysis of apoptotic cells was carried out as described previously (Radha et al., 1999; Shivakrupa et al., 2003; Subhash et al., 2006). For estimation of apoptosis cells were plated on cover slips and required transfections or treatments were carried out. At appropriate time the cells were fixed and processed for immunostaining using appropriate antibodies for detection of expressing cells and mounted in DAPI containing mountant to observe nuclear morphology. Cells showing immunofluorescence staining were counted and those cells that showed loss of refraction, condensed chromatin, apoptotic bodies and cell shrinkage were scored as apoptotic. At least 200 expressing cells were counted from each cover slip. The data represent the mean ± S.D. from at least three independent experiments on duplicate coverslips. Background apoptosis was determined by counting non-expressing cells in the same coverslips.

TUNEL assay was carried out as described previously (Shivakrupa et al., 2003). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay was performed using In Situ Cell Death Detection Kit, TMR red, (Roche) according to the manufacturer’s specification. Cos-1 cells on coverslips were transfected with control plasmid or c-Abl and 30 hrs later fixed in 4% formaldehyde in PBS for 1 hour. Cells were permeabilized in cold PBS with 0.1% Triton X-100 and 0.1% sodium citrate for 2 min in ice. Coverslips were washed thrice in PBS and 50 µl of TUNEL reaction mix (previously prepared & kept in ice till use as: 10 µl enzyme solution + 90 µl label solution) was added and left for 60 min at 37°C in a humidified dark chamber. After washing thrice in PBS, coverslips were mounted and observed under a fluorescence microscope. After TUNEL labeling, c-Abl transfected cells were stained with Abl antibody and conjugated secondary antibodies to visualize the transfected cells and confirm TUNEL positivity of cells that show apoptotic morphology.

2.2.25. Sequence analysis

NCBI programs BLASTn and BLASTp were used to analyze sequence similarity and database search. Bl2seq was used to align two nucleotide sequences based on their similarity (http://www.ncbi.nlm.nih.gov/BLAST/).
WebCutter 2.0 was used for analyzing restriction enzyme sites. Sequence Manipulation Suite was used to arrange and reverse complement sequences (http://www.ualberta.ca/~stothard/javascript/).

2.2.26. Statistical analysis

All data obtained was tested for levels of significance by the student’s t-test using Microcal Origin version 4.0 (microcal Software, Inc. USA). When significant differences were observed, P values for pairwise comparisons were calculated using the tailed t-test. P value less than 0.05 was considered significant.