CHAPTER 2
MATERIALS AND METHODS
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2.1 MATERIALS

Angiotensin II, Coomassie brilliant blue R-250, Epidermal growth factor, glucagon, glucose-6-phosphate, guanosine 5'-diphosphate (GDP), guanosine-5'-triphosphate (GTP), N-2-hydroxy ethylpiperazine-N'-2-ethane sulfonic acid (HEPES), N-lauryl sarcosine (Sarcosyl), 2-mercaptoethanol, Nonidet P40, phenyl methyl sulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS), sucrose, Tween-80 and vasopressin were obtained from Sigma Chemical Company, St Louis Missouri, USA.

Dextran T-500, protein-A, protein-A-Sepharose CL-4B, Sephadex G-25 and standard molecular weight markers were purchased from Pharmacia, Uppsala, Sweden. Guanosine-5'-O-2-thiodiphosphate (GDPβS) and V₈-protease (also called endoproteinase Glu-C) were obtained from Boehringer Mannheim GMBH, West Germany. Acrylamide, bisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED) were bought from Serva Fine Biochemica, Heidelberg, West Germany. 5'-guanylyl imidodiphosphate (GppNHp) was obtained from PL Biochemicals Inc, Milwaukee, USA. Nitrocellulose paper was obtained from Schleicher and Schuell, West Germany. X-ray films were obtained from Konica Corporation, Tokyo, Japan. Intensifying screens were purchased from DuPont, USA and Kasei Optonix Ltd., Tokyo, Japan. Complete Freund's Adjuvant and agar-agar were from Difco Laboratories, Michigan, USA.
Guanosine 5′-[γ-32P] triphosphate was obtained from Amersham International plc, UK. Adenosine 5′-[γ-32P] triphosphate was obtained from Bhabha Atomic Research Centre (BARC) Bombay and also from Jonaki, CCMB, Hyderabad, a unit of Board of Radio Isotope Technology. [32P] Phosphoric acid and I125 were obtained from BARC, Bombay. All other chemicals were obtained locally and were of analytical grade.

RAS 1 (viral Harvey ras); EC (normal human c-ras), EJ (oncogenically activated ras), EC/v-Ha (EC with 3′ region of Harvey ras), EJ/v-Ha (EJ with 3′ region of Harvey ras) (Gibbs et al., 1984a; Stein et al., 1984) were generously supplied by Drs M Poe and J B Gibbs of Merck Sharp and Dohme Research Laboratories, West Point, Pennsylvania, USA. EF-Tu (laCour et al., 1985) was a gift by Dr B F C Clark of Aarhus University, Denmark. nef-HIV1 (Guy et al., 1987) was kindly provided by Transgene. Transducin (Yatsunami et al., 1985) was a gift from Dr H G Khorana, Massachusetts Institute of Technology, Cambridge USA. The liver tumours induced by 2-acetyl aminofluorene (AAF), alfatoxin and ciprofibrate (Rao et al., 1988) were provided by Dr J K Reddy, North Western University, Chicago, USA. The antibodies against the subunits of G proteins (Mumby et al., 1986) were a gift from Dr A G Gilman University of Texas, Dallas, USA. The adult and fetal human liver tissues were obtained from a local hospital.

2.2 METHODS

2.2.1 Preparation of plasma membranes

The biphasic method described by Lesko et al. (1973) was used for preparation of plasma membranes from rat liver. The same method
was used for the preparation of fetal- and regenerating liver. The cells from regenerating liver were collected 48 h after partial hepatectomy.

Rat liver (Wistar) was perfused with PBS, excised, minced and homogenised in ten volumes of bicarbonate buffer (1mM NaHCO$_3$, 0.5mM CaCl$_2$ and 1mM PMSF, pH 7.4) by 20 up and down strokes in a loose fitting Dounce homogeniser. A light microscope was used to monitor cell lysis. The homogenate was centrifuged at 1,500xg for 10 min. The nuclei formed a tight pellet above which the membranes sedimented in a loose fluffy layer. The supernatant was poured off and the membrane pellet was resuspended in the starting volume of bicarbonate buffer. The centrifugation step was repeated 5 times. At this stage the supernatant was clear and nuclear pellet was no more visible. The crude membrane fraction thus obtained was purified on a dextran-polyethylene glycol biphasic. The biphasic was prepared as follows: 200g of 20% w/w dextran T-500, 103g of 33% w/w polyethyl glycol 8000, 333ml of 0.22M sodium phosphate-buffer pH 6.5 and 179ml distilled water were mixed in a separating funnel and allowed to settle at 4°C for 48 h. The top and bottom phases were collected separately. The membrane fraction was mixed with the top phase and 10 ml of the suspension was layered over 10ml of the bottom phase in 50ml Nalgene tubes. The two phases were mixed by gentle swirling and the tubes were centrifuged in a swing bucket (HB4) rotor at 1,100xg for 15 min. The material at the interface was collected and diluted with bicarbonate buffer and was centrifuged at 15,000xg for 15 min. The pellet was resuspended in the top phase and again purified on the biphasic as before. The final plasma membrane pellet was suspended in 10mM HEPES pH 7.5 containing 1mM PMSF and was frozen in aliquots
at -20°C. The assays for checking purity of the membrane fraction have been described earlier (Shashikant et al., 1983). The same method was used to prepare plasma membranes from ZAH cells except for the fact that 2mM CaCl₂ was used in bicarbonate buffer and the cells were incubated in bicarbonate buffer for 20 min and the cells were incubated in bicarbonate buffer for 20 min on ice prior to homogenization.

2.2.2 Preparation of mitochondria

Mitochondrial membranes were prepared essentially as described by Greenawalt (1974). Livers were excised from adult Wistar rats and minced and rinsed twice with the isolation medium. The isolation medium (pH 7.4) consisted of 0.07M sucrose, 0.21M D-mannitol, 2mM HEPES, 1mM EDTA and 1mM PMSF. A suspension was made of one volume of minced liver plus two volumes of isolation medium. The suspension was homogenized in a glass homogenizer. The homogenate was diluted 1:3 with isolation medium centrifuged at 660xg in a swing bucket rotor for 10 min. The supernatant fluid was carefully removed so that no loosely packed material was removed. Then the supernatant was transferred into ice-cold Sorvall tubes and centrifuged for 15 min at 7000xg in the Sorvall SS-34 rotor. Resulting supernatant was discarded. The pellet was suspended in one half of the original volume of the homogenate prior to the initial centrifugation. This suspension was then centrifuged for 15 min in the Sorvall SS-34 rotor at 7000xg. The resulting supernatant fluid was decanted and any resulting fluffy layer on the pellet was carefully rinsed off. The pellet was suspended in one-fourth of the original volume of the homogenate prior to initial centrifugation and again centrifuged for 15 min at the above speed. The supernatant was again decanted.
The pellet contained the mitochondria.

The mitochondrial fraction obtained as described above was further purified by the procedure of Bogenhagen and Clayton (1974). The mitochondrial pellet was suspended in 5ml of mannitol-sucrose buffer (0.21M D-mannitol, 0.07M sucrose, 5mM Tris pH 7.5, 5mM EDTA) and layered over a discontinuous sucrose gradient consisting 15ml of 1.0M sucrose 5mM EDTA, 10mM Tris pH 7.5 on top of 15ml of 1.5M sucrose, 5mM EDTA, 10mM Tris pH 7.5. The tubes were centrifuged for 30 min at 60,000xg in a Beckman SW28 rotor. The mitochondria were collected from 1.0 to 1.5M sucrose interface by pipetting, diluted with two volumes of mannitol sucrose buffer and pelleted by centrifugation as above.

2.2.3 Phosphorylation reactions

The phosphorylation reactions were carried out at 30°C in a 100μl reaction mixture comprising 1mM HEPES at pH 7.5, 0.21M D-mannitol, 0.07M sucrose, 2.5mM MgCl₂ and 20μg liver plasma membrane or ZAH plasma membrane where indicated. ras, EF-Tu, nef-HIV1, transducin, GDP, GTP, GppNHp or the factors like angiotensin II, epidermal growth factor, glucagon and vasopressin were added as required for individual experiments. The reactions were started by adding 10μCi [γ-32P] ATP (3000Ci/mmol) and were terminated with the addition of 50μl electrophoretic sample buffer (to give final concentrations of 0.0625M Tris, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% Bromophenol blue). In some experiments 1μM [γ-32P] GTP (~10Ci/mmol) was used instead of radiolabelled ATP. The samples were then boiled at 100°C for 5 min and analyzed by discontinuous SDS gel electrophoresis (Laemmli,
1970; also see below) with 10% acrylamide in resolving gel. The gels were fixed in a solution containing 10% isopropyl alcohol and 10mM sodium pyrophosphate, washed several times with the same solution dried and autoradiographed. For autoradiography the dried gels were usually exposed to X-ray films at -70°C for 12 h unless otherwise stated. In order to determine the lability of the phosphate moieties gels were treated overnight with a solution containing 15% (v/v) acetic acid and 15% (v/v) methanol. Quantitation of the intensities of the bands on the autoradiograms were done using a soft laser scanning densitometer (Biomed Instruments, California).

Phosphorylation reactions with AlF$_4^-$ were performed under the same conditions as described above, except for the fact that the liver plasma membranes were preincubated with 20μM AlCl$_3$ + 5mM NaF for 50 min at 20°C (Sternweis and Gilman, 1982).

### 2.2.4 Solubilization and phosphorylation of mitochondrial proteins

The experiments were carried out as described by Backer and Weinstein (1986b). The mitochondrial proteins were solubilized by incubating the mitochondrial fraction in 10mM glucose-6-phosphate at 20°C and centrifugation at 100,000xg for 1 h. The supernatant was used for phosphorylation studies.

The phosphorylation reactions with solubilized mitochondrial proteins were carried out in a 100μl reaction mixture containing 0.21M D-mannitol, 0.07M sucrose, 1mM HEPES (pH 6.8), 2.5mM MgCl$_2$, 10mM NaF and 20μg of mitochondrial proteins with or without ras proteins.
The phosphorylation reactions, subsequent electrophoresis and autoradiography with or without prior treatment of gels with acid were carried out as described above for liver plasma membrane proteins.

2.2.5 Peptide mapping

The phosphopeptide maps were obtained using the procedure described by Cleveland et al. (1977). The gels for this purpose were cast with longer than usual stacking gels (upto 5cm). In the gel solutions 1mM EDTA was included as recommended in the procedure. After phosphorylation reactions, electrophoresis and autoradiography the p38-bands were excised from the dried gels, trimmed to 5mm width and then soaked for 30 min with occasional swirling in 10ml of a solution containing final concentrations of 0.125M Tris/HCl, pH 6.8, 0.1% SDS and 1mM EDTA. The final sample wells were filled with the same buffer and each gel slice was pushed to the bottom of a well with a spatula. Spaces around the slices were filled by overlaying each slice with 10μl of this buffer containing 20% glycerol. Finally 10μl of this buffer containing 10% glycerol and a given amount of protease was overlayed into each slot and electrophoresis was performed in a normal manner with the exception that the current was turned off for 30 min when the bromophenol blue neared the bottom of the stacking gel. The resolving gel usually contained 15% acrylamide. After electrophoresis the gels were fixed in a solution containing 10% isopropyl alcohol and 10mM sodium pyrophosphate washed several times with the same solution, dried and autoradiographed. The autoradiography was done by exposing the dried gels to X-ray films at -70°C for 4-6 days.
2.2.6 Electroelution of p38

The purification of p38 from Coomassie blue-stained gels was carried out according to the procedure described by Hunkapillar et al. (1983). After SDS-PAGE and staining with Coomassie blue the p38-bands were excised and trimmed to approximately 1mm cubes with a sharp razor blade. The gel pieces were soaked in elution buffer (0.1% SDS in 0.05M NH$_4$HCO$_3$) for 5 min and the buffer was removed. The pieces were put into the cups of the elution apparatus.

The arrangement is described below. The cups have two wells: one for holding the gel pieces; the other for collection of eluted proteins. The distance between these two cups is such that it fits snugly on the ridge of the elution chamber. Both the wells have appropriate dialysis membranes fitted on them. The wells dip into the elution buffer (0.1% SDS in 0.05M NH$_4$HCO$_3$) on either side of the ridge. The contact between the two sides of the ridge is only through the buffers in the cups. The gel pieces are oriented towards negative electrodes and the collection chamber is towards the positive electrode. The elution of proteins occurs electrophoretically and can be monitored by visual observation of Coomassie blue which gets collected in the sample collection well.

After placing the pieces of the gel in the well they were overlaid with 2% SDS in 0.4M NH$_4$HCO$_3$. The elution proceeded at room temperature for 24 h. The sample collected in the wells was collected into glass-centrifuge tubes. The SDS and Coomassie blue were removed by precipitation of the protein in cold acetone. This was done by adding two volumes of cold acetone to the sample and incubation at -20°C for
48 h. The precipitates were collected by centrifugation at 10,000xg for 20 min.

### 2.2.7 Antisera against p38

The protein (p38) purified by electroelution was dissolved in PBS and emulsified in Complete Freund's Adjuvant (CFA) and injected on the backs of New Zealand White rabbits intradermally at multiple sites. Approximately 100μg protein was given in each injection. The booster doses were given in Incomplete Freund's Adjuvant (IFA). After three weeks the rabbits were bled for testing by Ouchterlony double diffusion method. When the test was positive with pure p38 the rabbits were bled through the eye-sinus, the sera were collected and stored after the addition of 0.1% sodium azide as preservative.

### 2.2.8 Ouchterlony double diffusion analysis

The test bleedings were monitored by the method described by Ouchterlony (1962). In order to do this an agar solution (1%) was prepared in PBS containing 0.1% sodium azide. The solution (3ml) was spread evenly on a glass slide (2cm x 4cm) and left at room temperature for solidification. Wells were cut in the agar using a well former from LKB instruments. The antigen and antibody solutions were filled in the wells. The slides were incubated in a moist chamber. Formation of an opaque precipitin line indicated positive reaction of antigen and antibody.

### 2.2.9 Western blots

The transfer of proteins resolved by SDS-PAGE was carried out according to the procedure of Towbin et al. (1979). The blottings were
performed with a Hoefer-Transphor apparatus. The transfer-buffer contained 0.192M glycine, 0.025M Tris, pH 8.3 and 20% methanol. The nitrocellulose paper was soaked in water and the transfer-buffer sequentially. After the electrophoresis, the gels were equilibrated in the transfer buffer after which the wet nitrocellulose paper was placed on the gel. All the air bubbles between the gel and the nitrocellulose paper were removed. The gel and the nitrocellulose papers were sandwitched between layers of Whatman No.3 filter paper soaked in the transfer buffer. This was in turn placed between two foam pads wetted in the transfer buffer. The whole assembly was put in a cassette with grids (provided with the instrument) and placed in the tank. The transfer was carried out for 3 h at 300mA. A cooling system was provided by circulation of water at 15°C from a water bath. The efficiency of transfer was monitored by staining a strip cut from the nitrocellulose paper with amido black (1% in 7% acetic acid).

The Western blots were done by following the procedure described by Gould et al. (1984) with minor modifications. After the transfer, the blots were rinsed with PBS, incubated for 15 min in buffer A (PBS, 0.25% gelatin, 0.1% Tween 80, 0.018 sodium azide) and for 3 h in buffer A containing 1% horse serum. After three washings with buffer A the blots were incubated overnight with anti-p38 antibody (1:100 dilution made in buffer A) in plastic bags. The blots were washed five times with buffer A before incubating overnight with \(^{125}\text{I}-\text{Protein A (made in buffer A Specific activity 10\mu Ci/\mu g diluted to 1\mu Ci/ml). Then the blots were successively washed over a period of 4 h, five times with buffer A, five times with buffer A containing 0.4% sarcosyl, five times with
buffer A containing additional 150mM NaCl and again five times with buffer A. The blots were then air dried and exposed to X-ray films overnight at -70°C. The intensities of the p38 band were measured using a soft laser scanning densitometer (Biomed Instruments, California).

The Western blot experiments with antibodies against the subunits of G proteins were performed in the same way except for the fact that they were used at different dilutions as required for individual experiments (Mumby et al., 1986). The yeast extract for the Western blot and other experiments was made from spheroplasts which were prepared according to the methods of Casperson et al. (1983).

2.2.10 Preparation of \( \text{I}^{125} \)-labelled Protein-A

Radioiodination of **Staphylococcus aureus** Protein-A was done using the chloramine T-method (Hunter and Greenwood, 1962). Radioactive iodine was obtained as NaI\(^{125} \). A typical reaction was carried out as follows: 1mCi of iodine was used for iodination of 1mg protein A. To 25\( \mu \)l iodine solution equal volume of 0.2M \( \text{NaH}_2\text{PO}_4 \) was added. To this, required quantity of protein A dissolved in PBS was added. Reaction was started by adding 10\( \mu \)g chloramine T dissolved in PBS. The reaction mixture was incubated at room temperature for 10 min. Reaction was terminated by the addition of 10\( \mu \)l (1mg/ml in PBS) sodium metabisulphite. The mixture was immediately loaded onto Sephadex G-25 column (made in a 10ml disposable pipette) and 30 fractions of 0.5ml each were collected. 1\( \mu \)l was taken from each fraction and counted in a Hewlett Packard gamma counter. The fractions in the first peak which consisted of protein A were pooled and stored at -20°C.
2.2.11 Immunoprecipitation

Immunoprecipitation experiments were performed essentially according to the procedure described by Davis et al. (1986). In order to do this, phosphorylation reactions were carried out as described earlier with the exception that they were terminated with 50μl of 3x immunoprecipitation buffer (1x corresponds to 10mM Tris pH 7.4, 1mM PMSF, 1% Nonidet P40, 2mM EDTA and 0.15M NaCl) and rapid cooling in crushed ice. To this 50μg of bovine serum albumin was added to raise the total protein concentration. To a 300μl aliquot taken in an eppendorf tube 2μl normal rabbit serum was added and incubated for 10 min at room temperature. 40μl protein A-Sepharose was added to each tube and the tubes were rocked gently at 4°C for 30 min. The protein A-Sepharose was collected by spinning in a microcentrifuge for 1 min. The pellet was discarded and the supernatants were transferred to new tubes. To one set of tubes 2μl normal rabbit serum was added and to the other 2μl of anti-p38 sera were added at the required dilution (usually 1:100). These tubes were incubated overnight at 4°C. Then 80μl protein A-Sepharose was added to each of these tubes and incubation was at 4°C for 30 min with gentle rocking. These tubes were spun in a microcentrifuge for 1 min. The supernatant was discarded. Each of the pellets was washed with 300μl immunoprecipitation buffer. After shaking gently the tubes were spun for 1 min in a microcentrifuge. The supernatant was discarded. Then to each of these pellets 40μl electrophoretic sample buffer was added and the tubes containing the pellets were placed in boiling water for 2 min. After spinning for 5 min in a microcentrifuge the supernatants were subjected to SDS-PAGE with a 10% resolving gel. After electrophoresis the gels were fixed in a solution containing 10% isopropyl alcohol.
and 10mM sodium pyrophosphate, washed with the same solution several times, dried and autoradiographed.

2.2.12 Metabolic labelling of liver cells with $^{32}$P

The liver cells were grown in suspension (Berry and Friend, 1969; Garrison and Haynes, 1975) in phosphate-free Dulbecco Modified Eagle Medium ($1 \times 10^6$ cells/ml containing 1mCi/ml carrier free $^{32}$P). The cells were incubated at 37°C for 90 min to label the intracellular nucleotides. To check the in vivo of stimulation of phosphorylation of p38, glucagon (20 µg/ml) was added at the end of the incubation and the cells were further incubated for 5 min. The incubation was terminated by rapid cooling in crushed ice and the cells were immediately collected by centrifugation, washed and lysed in immunoprecipitation buffer and the immunoprecipitations were carried out as described earlier.

2.2.13 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to method described by Laemmli (1970). Usually the separating gel had 10% acrylamide, 0.375M Tris.HCl pH 8.8, 0.1% SDS. The gel was polymerized using 125 µl of 10% (w/v) ammonium persulphate and 25 µl TEMED. The stacking gel which contained 4.5% acrylamide, 0.125M Tris.HCl pH 6.8 and 0.1% SDS was polymerized using 50 µl 10% ammonium persulphate and 15 µl TEMED. The protein samples were mixed with sample buffer (0.625M Tris.HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% Bromophenol blue). Electrophoresis was carried out using constant current using a buffer in the tank which comprised 0.025M Tris, 0.192M glycine pH 8.3 and 0.1% SDS.
After electrophoresis of products of phosphorylation reaction when the gels were stained to visualize phosphorylated p38, a staining method which does not employ acid was used. According to this method (Fujitaki et al., 1981) gels were stained with 0.1% Coomassie blue made in 25% isopropyl alcohol made alkaline with NaHCO₃ (pH 8) and were destained with 10% isopropyl alcohol adjusted to pH 8 with NaHCO₃.

Acid treatment of the gels was accomplished by treating the gels overnight with a solution containing 15% (v/v) acetic acid and 15% (v/v) methanol. Staining and destaining of the gels were done during the same period by including 0.2% Coomassie blue in the solution described above for the first 2 h.

In other circumstances the gels were stained with 0.2% Coomassie blue made in methanol : acetic acid : water (45:10:45) for 4 to 6 h. Destaining was carried out in the same solution without Coomassie blue.

The autoradiography was usually done as follows: The gels were fixed and washed as described under 'phosphorylation reactions'. Then the gels were dried at 80°C under vacuum in a Hoefer gel drier. The dried gels were exposed to X-ray films (Konica) with intensifying screens (Kyokko or DuPont at -70°C).

Protein estimations were done by a modified procedure of Lowry et al. (1951). To 0.2ml of protein solution 1ml of alkaline tartarate solution (2% Na₂CO₃, 0.2% Na, K tartarate and 0.002% CuSO₄ in 0.1N NaOH) was added. After 20 min incubation at room temperature, SDS (0.1ml of 0.5%) and Folin-Ciocalteau reagent (0.1ml of 2N) were added in quick successions with vortexing. After 30 min, absorbance at 740nm was measured. A standard curve was obtained using BSA.