Enhanced expression of heterotrimeric GTP-binding protein subunits in Zajdela ascitic hepatoma

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G protein beta subunit (Gβ2) is over-expressed in Zajdela ascitic hepatoma (ZAH), a rat ascitic tumour, at mRNA as well as protein levels. Nuclear run-off transcription analysis suggests that the expression of Gβ2 in ZAH is regulated mainly at transcriptional as well as post-transcriptional levels. Gα3 is also over-expressed in ZAH. No amplification or any change in the organization of the genes for Gβ2 or Gα3 was observed. It is possible that the over-expression of G protein subunits in ZAH could provide proliferative advantage to the cells by virtue of their effects on second messenger systems.

Regulation of cell-proliferation and changes in the signal transduction machinery upon transformation has been the subject of immense interest. A variety of cell surface receptors mediate their actions through heterotrimeric GTP-binding proteins (G proteins) that are made up of alpha, beta and the gamma subunits and function as intermediaries in the signal transduction machinery consisting of receptor, G protein and the effector. G proteins are inactive in the GDP bound state and active in the GTP bound form. The dissociated α and βγ subunits interact with the effector molecules. Since in many cases G proteins generate signals involved in cell growth, their genes have the propensity to be converted into oncogenes. Alternatively, changes in the expression of G protein subunits may affect cell proliferation by virtue of their effects on second messenger systems.

Activating mutations in G protein subunits have been identified in human endocrine tumours. For example, Gα13 is activated by a mutation in pituitary tumours with elevated cAMP levels and growth hormone production. Studies involving mutations in Gα13 in human tumours and with cell-lines have shown that several G protein alpha subunits may be involved in cell transformation. The role of G protein βγ subunits in the process of transformation has not been studied in detail. Gβγ regulate several second messenger-generating enzymes such as phospholipase C and adenylate cyclase. We have used Zajdela ascitic hepatoma (ZAH), a rat hepatoma, as a model system to examine the role of G protein subunits in transformation. This study demonstrates changes in the expression of G protein subunits in ZAH in comparison with its normal counterpart, the rat liver.

Materials and Methods

Tumour cell lines

Rat hepatoma cell lines, ZAH C and ZAH D were originally obtained from D F. Zajdela, Institut de Radium, Orsay, France. These tumours were induced with a chemical carcinogen, dimethylaminoazobenzene in female and male Sprague Dawly rats, respectively. The tumour cell lines are maintained in the peritoneal cavity of rats by serial transplantation.

RNA isolation and Northern blot analysis

Total RNA from rat liver and ZAH cells was isolated as described by Chomczynski and Sacchi, fractionated on 1% denaturing agarose gels and transferred onto Hybond N membranes (Amersham). A 725 bp fragment of beta subunit of G proteins (Gβ2) was amplified by polymerase chain reaction from rat liver cDNA library (Stratagene) using primers: forward primer, 5'-ATC GAA TTC * AAG ATC TAC rrr GCC ATG CAC TG G G-3'; reverse primer, 5'-ATC GAA TTC * TCC CAG G CAC TG A C CTT CAG T TG A AG TCG-3'. The sequence before the asterisks represents the EcoRI site with additional bases to facilitate subsequent cloning. The

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Abbreviations: PMSF, phenyl methyl sulfonyl fluoride; PLC, phospholipase C; IP3, inositol 1, 4, 5, trisphosphate; DAG, diacylglycerol; MAP kinase, mitogen activated protein kinase.
PCR product was cloned in EcoRI site of the bacterial vector, pBS. Sequencing of this clone confirmed its identity as Gβ2. Gβ2 sequence excised from this clone was labelled using \([\alpha^{-32P}]dATP\) and multiprime labeling kit and used as a probe in hybridization. The labelled DNA was separated from unincorporated nucleotides using spun column chromatography\(^{11}\). Blots were prehybridized for 2-4 hr and hybridized at 65°C overnight. Final washing of the blots was carried out at 65°C for 15-30 min with 0.1×SSPE containing 0.1% SDS before exposing to autoradiography films.

Preparation of cell extracts

Rat liver was perfused with phosphate buffered saline (PBS; 10 mM Na\(_3\)HPO\(_4\), 2 mM KH\(_2\)PO\(_4\), 137 mM NaCl, 2.7 mM KCl), washed with cold PBS and homogenized in a loose fitting homogenizer by 3-4 strokes. The homogenate was diluted two fold with PBS and filtered through two layers of cheesecloth. The filtrate was centrifuged at 1500 rpm for one minute in a Remi tabletop centrifuge. The cell pellet was washed twice with PBS. For collection of ZAH cells, ascitic fluid was collected from the tumor bearing rats on the fifth or sixth day of transplantation, diluted with PBS and cells were recovered by centrifugation at 1500 rpm for one minute in a Remi table top centrifuge. Cells were washed 2-3 times with PBS. Liver or ZAH cells thus isolated were homogenized in PBS containing 1 mM PMSF using Polytron (Kinematica) at a speed setting of 5 for 2 min in cold condition. The homogenates were centrifuged at 1500 rpm for 5 min to remove the unlysed cells and stored at -70°C.

Separation of proteins by SDS-PAGE and Western blot analysis

Proteins were separated by SDS-PAGE on 10% polyacrylamide gels as described by Laemmli\(^{12}\) and electrophoretically transferred to nitrocellulose membranes\(^{13}\). After transfer, the membranes were washed with buffer A (PBS, 0.25% gelatin, 0.01% Tween 80, 0.01% sodium azide) and incubated for 1 hr at room temperature with buffer A containing 1% horse serum. Gβ antibodies (NEP-DuPONT) were diluted in buffer A and incubated with the membranes overnight. Membranes were washed with buffer A (3 times, 10 min each) then with buffer A containing 150 mM NaCl (once, 15 min). The binding of the primary antibody was detected using \([^{125}\text{I}]\)-Protein A. Radiiodination of Protein A from Staphylococcus aureus was carried out as described\(^{14}\). Membranes were exposed to films (Konica) for autoradiography.

Nuclear run-off transcription assay

Nuclear run-off transcription assay was carried out essentially as described\(^{15}\) using \([\alpha^{-32P}]UTP\). Equal number of nuclei from liver and ZAH C cells were used. Radio-labeled RNA after the run-off transcription was isolated as described by Chomczynski and Sacchi\(^{10}\). DNA dot-blot were prepared using rat Gβ2 cDNA. Plasmid DNA was used as a control. The dot-blot were prehybridized for 2-4 hr and then hybridized with the radio-labeled RNA at 65°C for 36 hr. The blots were washed with 2× SSPE at room temperature for 45 min followed by washing at 65°C for 60 min. The blots were then treated with RNase A (10 μg/ml in 2× SSPE) for 40 min at 37°C, washed with 2× SSPE at 37°C for 45 min and exposed to the autoradiography films. The blots were aligned with the autoradiogram, the “spots” were cut out and their associated radioactivities were counted in the scintillation counter.

Miscellaneous

All chemicals were purchased from commercial sources and were of analytical or molecular biology grade. All experiments were performed at least three times and data from a representative experiment are presented. Plasma membranes from liver and ZAH cells were isolated as described\(^{16}\) and stored at -70°C. Protein estimation was carried out as described by Lowry et al.\(^{17}\) using bovine serum albumin as standard. The relative intensities of the bands were measured by laser densitometer (Molecular Dynamics) using software (ImageQuant).

Results

Expression of G protein beta subunit in ZAH

We first examined if there were any differences in the steady state levels of Gβ subunit mRNA between rat liver and the ZAH cells. Total cellular RNA was fractionated on denaturing agarose/formaldehyde gels, transferred onto Hybond N membrane and hybridized with radio-labeled rat Gβ2 cDNA. The same blot was deprobed and probed with 28S rRNA gene. The results show that the steady state levels of Gβ2 mRNA in ZAH is about 6 fold more as compared to the liver (Fig. 1 A and B).

In order to examine if the higher steady state levels of Gβ mRNA resulted in higher steady state levels of the protein, we performed Western blot experiments
on cell extracts of liver, ZAH C and D cells using anti-Gβ antibodies. We observed that the steady state levels of Gβ protein in ZAH is about 4 fold more than that in the liver (Fig. 2A and B). We next examined if the over-expressed Gβ in ZAH was associated with the plasma membrane. Western blot experiments were performed on purified plasma membranes from liver and ZAH C cells. Again, the expression of Gβ was found to be about 5 fold more than that in the liver (Fig. 3A and B). The results for the first time demonstrate over-expression of Gβ in a chemically induced rat hepatoma.

**Relative rate of transcription of Gβ2 in liver and ZAH cells.**

The high steady state levels of Gβ2 mRNA in ZAH could be due to high transcription rate of the gene or due to its increased stability or both. Nuclear run-off transcription assay was performed on the nuclei isolated from liver and ZAH C cells to examine if there were any differences in the rate of transcription of Gβ2 gene. The results demonstrate that the relative rate of transcription of Gβ2 gene in ZAH C is about 3 fold more as compared to the liver (Fig. 4A and B).

**Expression of Gαi3 in ZAH**

Since the analysis of Gβ subunit showed that it is over-expressed in ZAH, it was of interest to examine if there were any changes in the expression of Gα subunit as well. Total cellular RNA was fractionated on denaturing agarose/formaldehyde gel, transferred onto Hybond N membrane and hybridized with radiolabeled Gαi3 cDNA (kindly provided by Dr R R Reed). The same blot was deprobed and probed with 28S rRNA gene. The results show a 2-3 fold increase in the expression of Gαi3 in ZAH (Fig. 5A and B).
Fig. 2—Gβ protein levels in liver and ZAH cells. (A): A total of 100 μg protein from liver and ZAH cell extracts was fractionated on 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane and probed with Gβ antibodies. Positions of Gβ and molecular weight markers (kDa) are indicated by arrows. (B): Relative levels of Gβ protein in liver and ZAH cells. L, liver; C, ZAH C and D, ZAH D.

Fig. 3—Association of over-expressed Gβ with plasma membrane in ZAH C cells. (A): A total of 100 μg protein from liver and ZAH C plasma membranes was fractionated on 10% SDS-polyacrylamide gel, transferred onto nitrocellulose membrane and probed with Gβ antibodies. Positions of Gβ and molecular weight markers (kDa) are indicated by arrows. (B) Relative levels of Gβ protein in liver and ZAH cells. L, liver; and C, ZAH C.
Fig. 4—Relative rate of transcription of Gβ2 gene in liver and ZAH cells. Nuclear run-off transcription on the nuclei isolated from liver and ZAH C cells was performed as described in the Materials and Methods. DNA dot-bLOTS were prepared using plasmid DNA (1) as control and rat Gβ2 cDNA (2). (A): The blots were hybridized with radio-labeled RNA, isolated after nuclear run-off transcription from liver and ZAH C nuclei. (B): Relative rate of transcription of Gβ2 in liver and ZAH C cells.

Fig. 5—Expression of Gα3 in liver and ZAH cells. Total RNA from liver and ZAH cells was size-fractionated on 1% denaturing formaldehyde-agarose gel, transferred onto Hybond N membrane and hybridized with radio-labeled Gα3 cDNA. (A): Expression levels of Gα3 mRNA. (B): Same blot was deprobed and then probed with 28S rRNA gene. (C): Relative levels of Gα3 mRNA in liver and ZAH cells after adjusting with the signal obtained with 28S rRNA gene. Lane 1, liver; lane 2, ZAH C and lane 3, ZAH D. Positions of Gα3 mRNA and ribosomal RNA bands are indicated by arrows.
Discussion

We have shown that G protein beta subunit is over-expressed in ZAH, a hepatic tumor at mRNA as well as protein levels. Over-expressed Gβ is associated with the plasma membrane making it capable of participating in the signal transduction pathways initiated by binding of the agonists to the cell surface receptors linked to G proteins. Nuclear run-off transcription assay on the nuclei isolated from liver and ZAH C cells revealed an increase of about 3 fold in the relative rate of Gβ2 transcription in ZAH. Since the steady state levels of Gβ2 mRNA in ZAH C is about 6 fold more than that in the liver, the results suggest that the expression of Gβ2 in ZAH C is regulated mainly at the transcriptional as well as post-transcriptional levels. Gα3 is also over-expressed in ZAH.

The functional significance of over-expression of G protein subunits in ZAH is not known at present. The Gβγ subunits, although not covalently linked to each other, are very tightly associated under physiological conditions and can be dissociated only with denaturants. Consequently, Gβγ subunits form a single functional unit in the cell. Since we have observed that over-expressed Gβ in ZAH is associated with the plasma membrane, it is possible that Gγ may also be correspondingly over-expressed and lipid-modified in ZAH. Gβγ activate many effectors including phospholipase C in ZAH. Many growth factors and oncogenes affect phospholipid metabolism that has been shown to be altered in transformed cells. Recently, Lalwani et al. have demonstrated that the chemically induced hepatic tumours possess elevated levels of second messengers, IP3 and DAG. Although we do not have any evidence at present, it is possible that the altered growth exhibited by ZAH, a chemically induced rat hepatoma, may at least in part, be due to the effect of Gβγ on PLC and consequent increase in the levels of IP3 and DAG. Alternatively, Gγ may provide proliferative advantage to ZAH cells by virtue of their effects on other effectors such as MAP kinase. Activation of MAP kinase has been implicated in cell growth and proliferation. Recently, G protein beta subunit has been shown to be required for normal growth in Aspergillus nidulans and a report by Ghahremani et al. has suggested a role for Gβγ in MAP kinase activation and DNA synthesis in BALB/c 3T3 cells.

Significance of over-expression of Gα3 in ZAH is also not known at present. Gα3 is involved in stimulation of phospholipase C. Thus, it is possible that over-expression of Gα3 may contribute to cell proliferation in ZAH through phosphoinositide pathway. Recently, Gα3 has been shown to be involved in transformation although the signalling pathway involved is not known. Further studies will be directed towards examining the signal transduction pathways affected by over-expression of the G protein subunits in ZAH.

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