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13. ABSTRACT *(Maximum 200 Words)*

Recent genetic understanding has identified the gene NF1 to be disrupted or mutated in patients affected with NF1 resulting in reduced expression of the protein neurofibromin. One function if NF1 has shown that it acts to negatively regulate, or turn off, positive signals in the cell that direct the cell to proliferate. Specifically, NF1 turns the positive signals relayed by a protein called Ras. However, other studies have shown that NF1 may negatively regulate cell proliferation by mechanisms other than through inactivation of Ras. These other mechanisms however are currently unknown. The purpose of the proposed study was to determine what changes in gene expression occur in cells from a patient with neurofibromatosis when we force these cells to express neurofibromin. The study was designed to identify and determine genes deregulated by NF1 in a Ras-dependent and Ras-independent manner. Taken together, these studies were designed to contribute to our understanding of the function and downstream effects NF1 has on the cell. In addition, determining the downstream nuclear events that occur in response to NF1 may provide novel targets for the treatment of NF1 associated cancers.

14. Subject terms

NF1, molecular biology, representational difference analysis

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INTRODUCTION

Neurofibromatosis type 1 (NF1) is a familial multisystem disease that occurs in approximately one in 3,500 people designating it as one of the most common autosomal-dominant human diseases. Approximately 5% of all NF1 patients develop malignant tumors making it the most common tumor-predisposing disease in humans [1]. Mutations in the *NF1* gene, which functions as a tumor suppressor, are considered to be the primary cause for the disease. NF1 is a very large protein composed of 2818 amino acids [2]. A small segment of the protein has been demonstrated to function as a negative regulator of the Ras family of small GTPases. However the biochemical functions of the remaining protein remains currently unknown. Thus, it seems likely that NF1 mediates its tumor suppressor activities through Ras-dependent as well as Ras-independent mechanisms. Therefore, we propose to determine what changes in gene expression occur as a result of loss of NF1 expression and to determine which expression changes are a consequence of Ras activity and which are not. We believe this information will lead to a more complete understanding of how NF1 mediates tumorigenesis and identify novel targets for the rational design of anti-cancer therapeutics against neurofibromatosis.

BODY

In specific aim 1, we proposed to determine if a collection of genes we previously identified as being aberrantly expressed as a result of Ras activity were also deregulated in a schwannoma cell line (ST88-14) that do not express NF1. To accomplish this, we first had to generate ST88-14 stable cell lines expressing full-length NF1 and NF1-GRD (Gap-Related Domain). We obtained a clone of mammalian NF1 in pBluescript (pBS) from Andre Bernards (Harvard) and tried several strategies for subcloning it into mammalian expression vectors. Based on information provided by Dr. Bernards, the full length clone is highly unstable when propagated in E.coli (memo enclosed in Appendix). Thus, our approach was to cut out and subclone the full-length NF1 insert from the pBS vector without propagating the plasmid. Our plan was to subclone the NF1 insert into two mammalian expression vectors; pBABE and pGFP-IRE. pBABE was chosen because it is a retroviral expression vector for efficient infection and it has puromyocin selection for fast, easy and clean selection. pGFP-IRE was chosen because it is a bicistronic mammalian expression vector driven by a CMV promoter in which both GFP and NF1 would be transcribed into one transcript, but by positioning NF1 behind GFP and having and internal ribosome entry (IRE) site between the two, most of the protein made off this transcript would be GFP with a small amount of NF1. This could reduce the potential problem of cellular toxicity of NF1 overexpression by standard expression vectors. This construct was chosen to minimize the amount of NF1 made in the cells since according the information provided by Dr. Bernards, cells do not tolerate NF1 very well. After ligation of the NF1 insert to both vectors and transformation of E.coli, we obtained several clones of NF1 in pBABE, but none in pGFP-IRE. After plasmid DNA purification and sequencing analysis of our pBABE/NF1 constructs we found that all of them contained deletions or mutations. One potential reason most of our plasmids

contained deletions is that our source of DNA is too old. To try to obtain new, full length NF1 cDNA we are currently attempting to PCR clone it using Clontech's Advantage 2 PCR kit which is designed to allow accurate cloning of long transcripts (NF1 is 8.5 kb in length). If this works, we will proceed with subcloning into the above mentioned vectors.

Gene deregulation due to loss of NF1 is thought to be mediated by both Ras-dependent as well as Ras-independent mechanisms [3, 4, 5, 6]. Ras has been demonstrated to be an important contributor to the process of transformation in tumors from NF1 patients. For example, Ras proteins are found in their active GTP-bound state in malignant tumor cells isolated from patients with NF1 [7, 4]. In addition, Ras-GTP levels are 5-fold higher in malignant as compared to benign neurofibroma tumors [3]. Forced expression of p120GAP (which inhibits Ras activity) or treatment with anti-Ras neutralizing antibody can reverse the transformed growth properties of ST88-14 cells [4, 7]. Taken together, this strongly suggests that aberrant Ras function is critical for the malignant transformation of NF1-deficient cancers. Thus, one approach to understanding some of the genes deregulated by loss of NF1 was to identify genes deregulated by Ras. Given the importance of Ras in NF1-mediated transformation, the potential of finding downstream targets for the development of therapeutics along with a better understanding of the process of transformation due to loss of NF1 is still maintained.

To identify those genes deregulated by loss of NF1 through Ras-dependent pathways, we first generated ST88-14 cells with decreased Ras-GTP levels. To do this, ST88-14 cells stably expressing p120GAP were generated. p120GAP is a GTPase Activating Protein which functions as an enzymatic stimulator of the intrinsic GTPase of Ras leading to the inactivation of Ras by converting Ras-GTP to Ras-GDP. ST88-14/p120GAP cells were generated by infecting cells with virus made with either the retroviral vector pLXSN/GAP or as control, vector only, and selected with G418 at 400ug/ml. As shown in Fig. 1, cells overexpressing p120GAP showed a reversion of the transformed phenotype seen in the parental cells. The cells expressing p120GAP appear less refractile and flatter compared to ST88-14 cells containing vector only. Confirmation of p120Gap overexpression was determined by western blot analysis (Fig.2) and showed a 2-3 fold increase in exogenous p120Gap expression relative to control vector-only cells. We next determined if the cells expressing p120GAP showed decreased Ras-GTP levels. To do this we performed Ras-GTP affinity precipitation pull-down assays. In this assay, only GTP-bound Ras is affinity purified from cell lysates which contain both GDP- and GTP-bound Ras. This is accomplished by use of a glutathione s-transferase (GST) fusion protein containing the Ras Binding Domain (RBD) of Raf-1 bound to glutathione agarose beads. The Raf-1 RBD is specific to and only binds GTP-bound Ras and not GDP-bound Ras. After the beads are mixed with protein lysates they are centrifuged and washed several times to remove all unbound protein. The beads are then boiled in 2x Laemmli sample buffer and the protein loaded onto a SDS-PAGE gel with subsequent detection of Ras by western blot. When performing the assay it is important to ensure that the cell lysates compared contain equal amounts of total protein. To do this we first determined protein concentrations of our lysates by standard Bradford protein determination assays and then followed these determinations by immunoblotting with total ERK antiserum (Fig.3) to confirm equal

protein levels . When Ras-GTP levels were compared (Fig.4), we found that, as expected, cells overexpressing p120GAP showed a decreased level of Ras-GTP when compared to that seen in the vector-only cells. Thus, the forced overexpression of p120GAP in the ST88-14 cells resulted in a decreased level of endogenous GTP-bound, active Ras. Interestingly, we also compared phospho-ERK (p42/p44 MAPK) levels as and indirect measure of Ras-GTP levels (Fig.5) and found no difference between cells overexpressing p120GAP and those containing vector only. Thus, although Ras-GTP levels are reduced in these cells, other mechanisms must exist in these tumor cells to maintain activation of ERK. Taken together, these data demonstrate that the cells expressing p120GAP result in lower Ras-GTP levels.

With the establishment of ST88-14 cells with reduced Ras-GTP levels, we examined whether a collection of genes we previously isolated by representational difference analysis (RDA) and showed to be deregulated by K-Ras(12V) in RIE-1 (Rat Intestinal Epithelial) cells [9] are also deregulated by Ras in the ST88-14 cells. Six of the genes from our original RDA screen were used in northern blot analyses of the ST88-14 cells with representative genes shown in Fig.6. We found that most of the genes showed no change in expression, like that seen for PTP-δ and tropomyosin-α, between the p120GAP expressing cells and those with vector only. The only gene for which we observed a small change in response to p120GAP expression was with transgelin. However, the increase in transgelin observed is very small (see Fig.6). Because we overwhelmingly saw no change in gene expression in this collection of genes we questioned whether the full-length p120GAP was fully biologically active. Our analysis of the GTP-Ras pull down assay (Fig.4) would suggest it was, but the data comparing phospho-ERK levels (Fig.5) would suggest it is not.

Since we were not convinced that the full-length p120GAP was fully biologically active, we decided to try using a N-terminal truncated version of p120GAP termed C-GAP to determine if it showed biological activity . C-GAP contains the catalytic region of GAP and is thought to have increased activity by truncation of a regulatory inhibitory domain at the N-terminus. Mass populations of ST88-14 cells stably expressing C-GAP were generated by retroviral infection (Fig.7). While these cells did appear to be slightly larger and flatter than parental cells (Fig.7), the change in morphology was not dramatic. When Ras-GTP levels were compared (Fig.9), we again found a decrease in Ras-GTP levels when C-GAP expressing cells were compared to vector only cells. While this was observed with the cells grown in 10% serum, it was not observed in the cells grown in 0.1% serum. In fact the cells grown in 0.1% serum actually showed an increase in Ras-GTP levels in the C-GAP expressing cells compared to vector only cells. However, while the total protein determinations of the lysates appeared to be equal (Fig.8), we believe the 0.1% pZIP Ras-GTP lane represents a technical error and is actually underloaded based on a comparison of the background bands on the blot. Thus, we conclude that the Ras-GTP data of the cells in 10% serum are representational and that C-GAP, like full-length GAP, causes a decrease in Ras-GTP levels. However, when phospho-ERK (p42/p44 MAPK) levels were compared (Fig.10), we again found no difference between cells with or without C-GAP overexpression.

Thus, while both full-length GAP and C-GAP appear to decrease Ras-GTP levels in the ST88-14 cells, other mechanisms appear to be capable to activating phospho-ERK. In addition, the change in Ras-GTP levels due to overexpression of GAP does not appear to be sufficient to cause a significant change in gene expression. Therefore, in order to directly address the changes in gene expression due to loss of NF1, restoration of full-length NF1 must first be accomplished. As mentioned above, we plan to try and clone full length NF1 cDNA from several human cell lines using Clontech's Advantage 2 PCR kit which is designed to allow accurate cloning of long transcripts (NF1 is 8.5 kb in length). Once accomplished, we will proceed with subcloning into the above mentioned vectors and repeat the above biological assays prior to a cDNA subtraction and chip array analysis.

KEY RESEARCH ACCOMPLISHMENTS

- Generation of ST88-14 cells stably expressing p120GAP and C-GAP
- Isolation of RNA from ST88-14 cells stably expressing p120GAP or vector only
- Isolation of mRNA and cDNA from ST88-14 cells stably expressing p120GAP
- Generation of all primers necessary to perform representational difference analysis

REFPORTABLE OUTCOMES

none thus far

CONCLUSIONS

We have completed specific aim 1 and found that while both full-length GAP and C-GAP appear to decrease Ras-GTP levels in ST88-14 cells, other mechanisms appear to be capable to activating phospho-ERK. These data may indicate that treatment approaches which attempt to utilize farnesyltransferase inhibitors (which inhibit Ras protein processing) may not be useful in these patients. In addition, the change in Ras-GTP levels in response to GAP overexpression in the ST88-14 cells does not appear to be sufficient to cause a significant change in gene expression due to Ras activation.

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APPENDICES

Dear Dr. Shields:

There are indeed major stability problems with human NF1 constructs. The problem lies in the ORF, since frame-shift mutations render constructs completely stable. This is such a strong effect that it has been virtually impossible for most people to grow intact NF1 expression vectors. What I do to circumvent this problem are a few things. First, I only use E. coli HB101 as a host. I know that other strains have similar genotypes as HB101, but nobody in my lab has ever been able to grow NF1 cDNAs in these strains. Secondly, any re-transformation of NF1 constructs gives many large and a smaller number of very small pinprick colonies. The large ones have acquired mutations (frame-shifts or deletions) that allow them to grow, and the small ones that don't grow are the ones you want. Don't ever try to grow NF1 cDNAs on plates that contain more than the minimum amount of antibiotics, or you will even more strongly select for the deleted clones. Finally, grow up small batches of the plasmids and only use those that grow very poorly (be very suspicious of any plasmid yield of >200 ug/liter). Before we stopped working on mammalian NF1, we routinely sequenced the entire 8.5 kb ORF of every new batch of plasmid to make sure that everything was OK.

In addition to the problem with growing NF1 constructs in E. coli, be also aware that mammalian cells don't tolerate any NF1 over-expression. Doug Lowy published a few papers on this charming property of the protein, which makes it even harder to do experiments that require NF1 transfection.

This said, I am fairly sure that I still have the last human NF1 construct I made, which was a full length UAS-driven Drosophila expression vector. I am not sure whether I have any mammalian expression vector available, but I'll let you know after I look.

My apologies for being longwinded, but I have send these clones to several people only to have them complain later that they were deleted and/or impossible to work with.

Best wishes,

Andre Bernards

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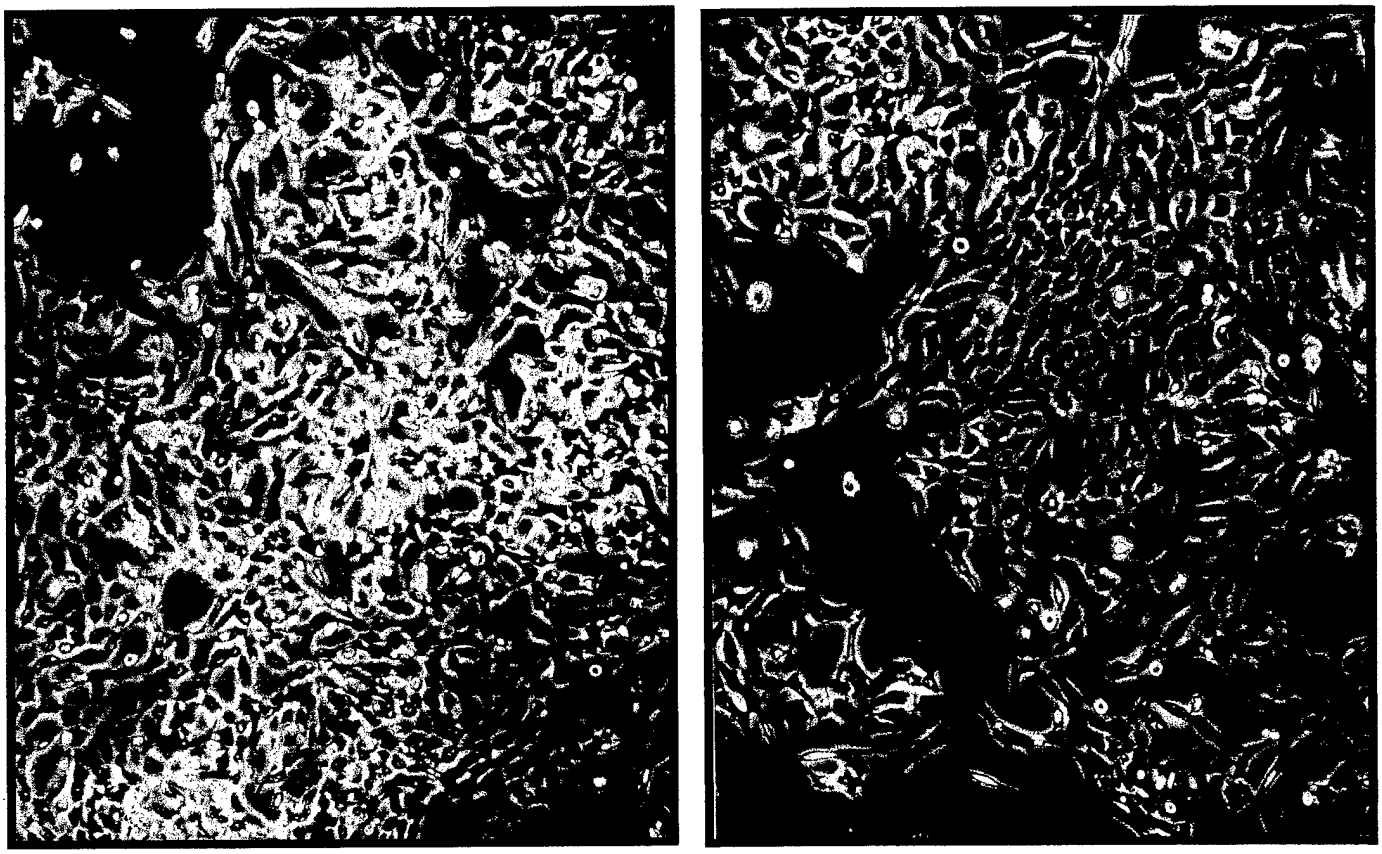
Fig.1. Morphology of Cells Stably Expressing p120GAP. Mass populations of ST88-14 cells stably expressing full-length p120 GAP were generated by retroviral infection. Cells were infected with virus generated with the pLXSN retroviral vector where expression of the inserted p120GAP gene is regulated by the 5' Long Terminal Repeat promoter and selected with 400 ug/ml G418. Shown on the left are pLXSN (empty vector) infected cells and on the right are pLXSN-p120GAP expressing cells. Note that the cells expressing p120GAP are less morphologically transformed than the control vector-only cells.



pLXSN **pLXSN / p120GAP**

Fig.2: Forced Overexpression of p120GAP in ST88-14 Cells. Equal amounts of protein lysates (30 ug) were prepared from ST88-14 cells stably expressing p120GAP, separated over a 8% SDS-PAGE gel, transferred to Immobilion-P, blotted with anti-GAP serum (Upstate) at 1 ug/ml and detected with HRP-conjugated secondary antibody with ECL. Shown are lysates from empty vector infected cells (pLXSN) and those expressing p120GAP (pLXSN/GAP) grown in low (0.1%) or normal (10%) serum.

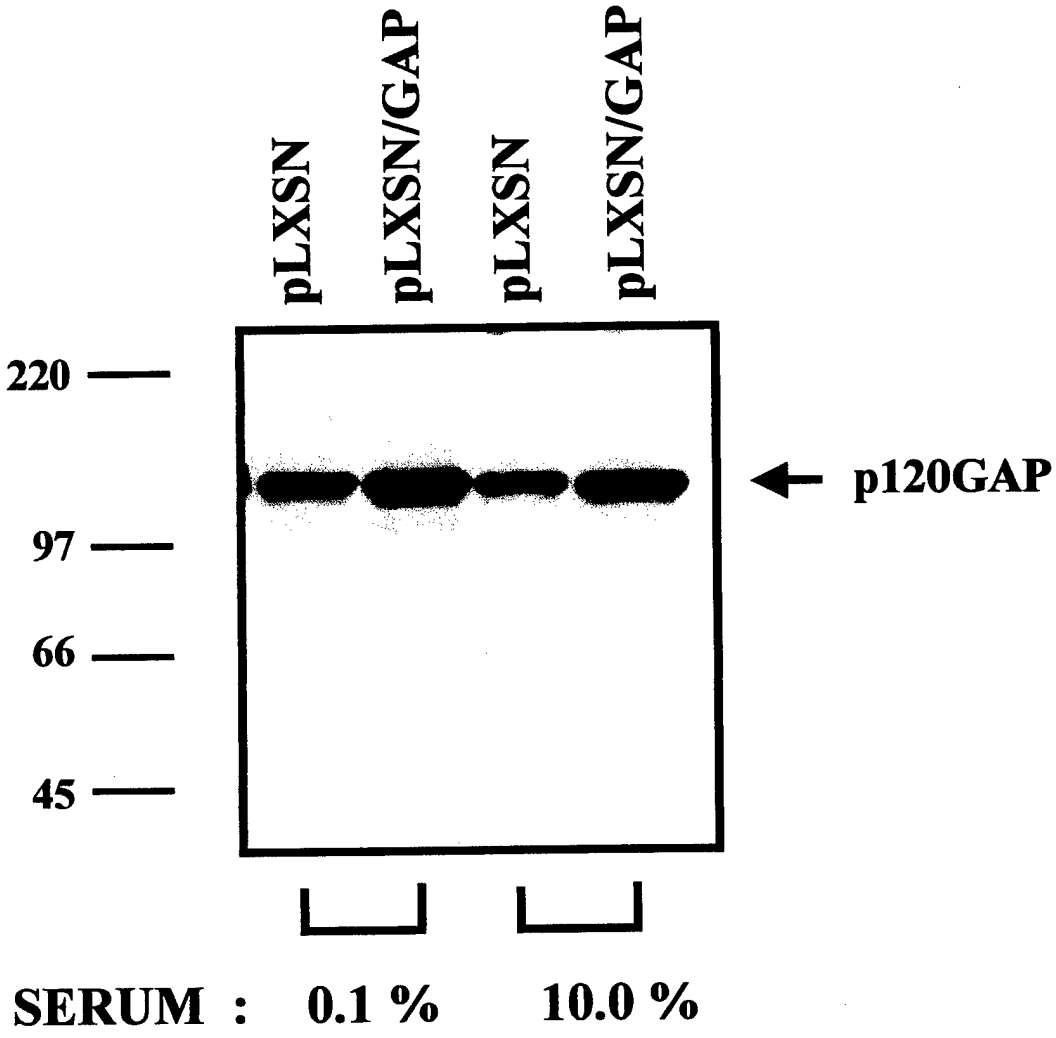


Fig.3: Total ERK Expression in ST88-14/p120GAP Cell Lysates. Equal amounts of protein lysates (30 ug) were prepared from ST88-14 cells stably expressing p120GAP, separated over a 12 % SDS-PAGE gel, transferred to Immobilion-P, blotted with anti-total ERK serum (Santa Cruz) at 1:1,000 and detected with HRP-conjugated secondary antibody with ECL. Shown are lysates from empty vector infected cells (pLXSN) and those expressing p120GAP (pLXSN/GAP) grown in low (0.1%) or normal (10%) serum.

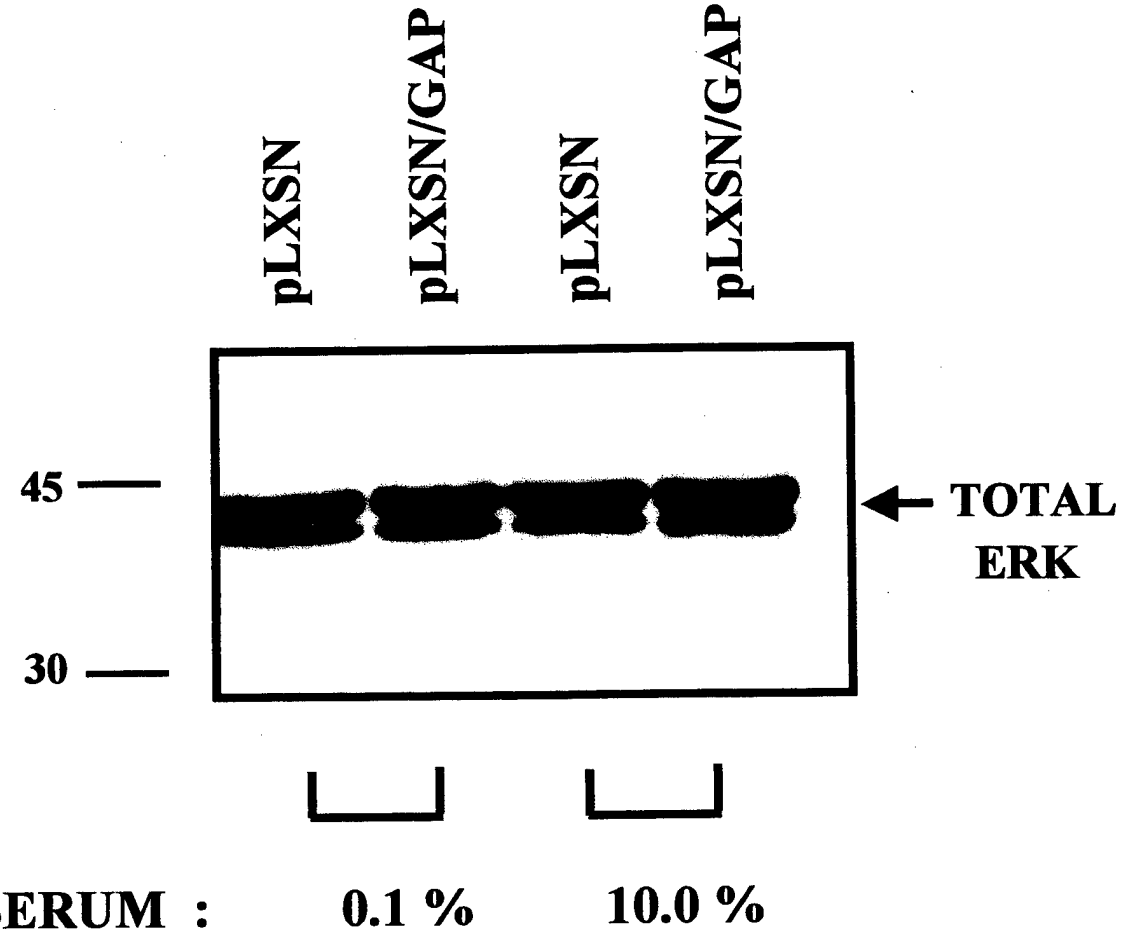


Fig.4: Ras-GTP Levels are Decreased in Cells Overexpressing p120GAP. Equal amounts of protein lysates (500 ug) prepared from ST88-14 cells stably expressing p120GAP or vector only were mixed with 5 ug Raf-1 RBD-agarose (Upstate), washed, separated over a 12 % SDS-PAGE gel, transferred to Immobilion-P, blotted with anti-pan-Ras serum (Oncogene Research Products) at 2.5 ug/ml and detected with HRP-conjugated secondary antibody with ECL. Shown are Ras-GTP levels from lysates of empty vector infected cells (pLXSN) and those expressing p120GAP (pLXSN/GAP) grown in 0.1% serum 24h .

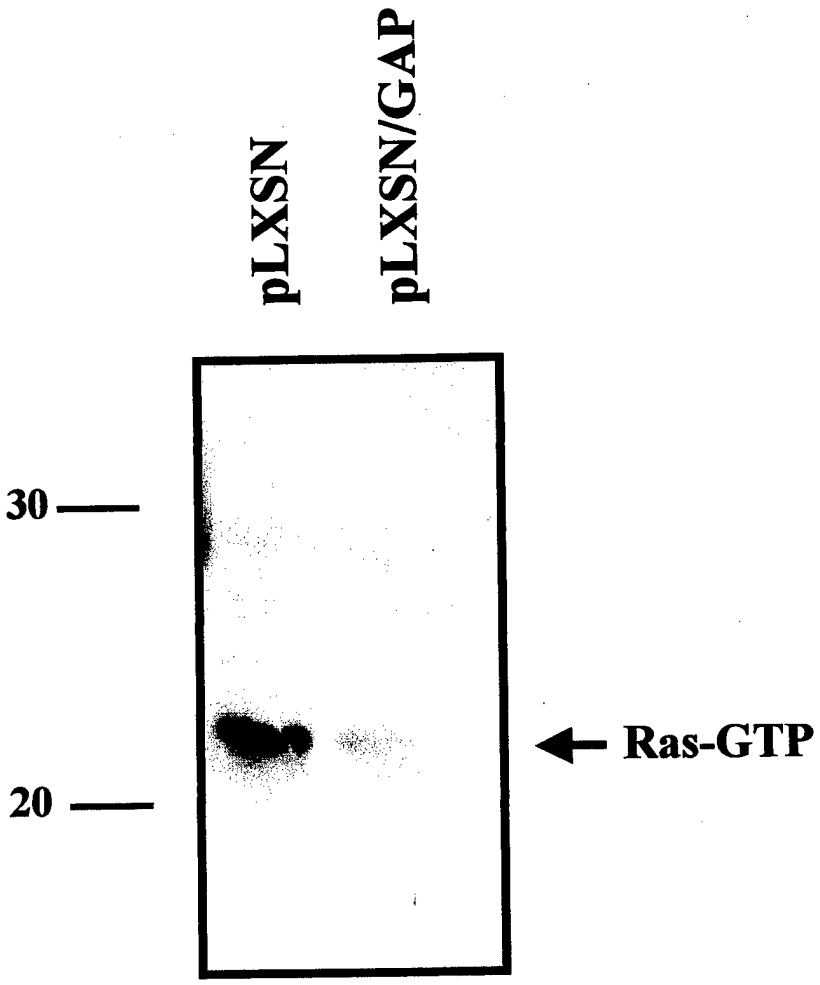


Fig.5: Phospho-ERK Levels are Not Altered in Cells Overexpressing p120GAP. Equal amounts of protein lysates (30 ug) were prepared from ST88-14 cells stably expressing p120GAP, separated over a 12% SDS-PAGE gel, transferred to Immobilion-P, blotted with anti-phospho-p44/42 MAPK serum (Cell Signaling) at 1:1,000 overnight and detected with HRP-conjugated secondary antibody with ECL. Shown are lysates from empty vector infected cells (pLXSN) and those expressing p120GAP (pLXSN/GAP) grown in low (0.1%) serum 24h.

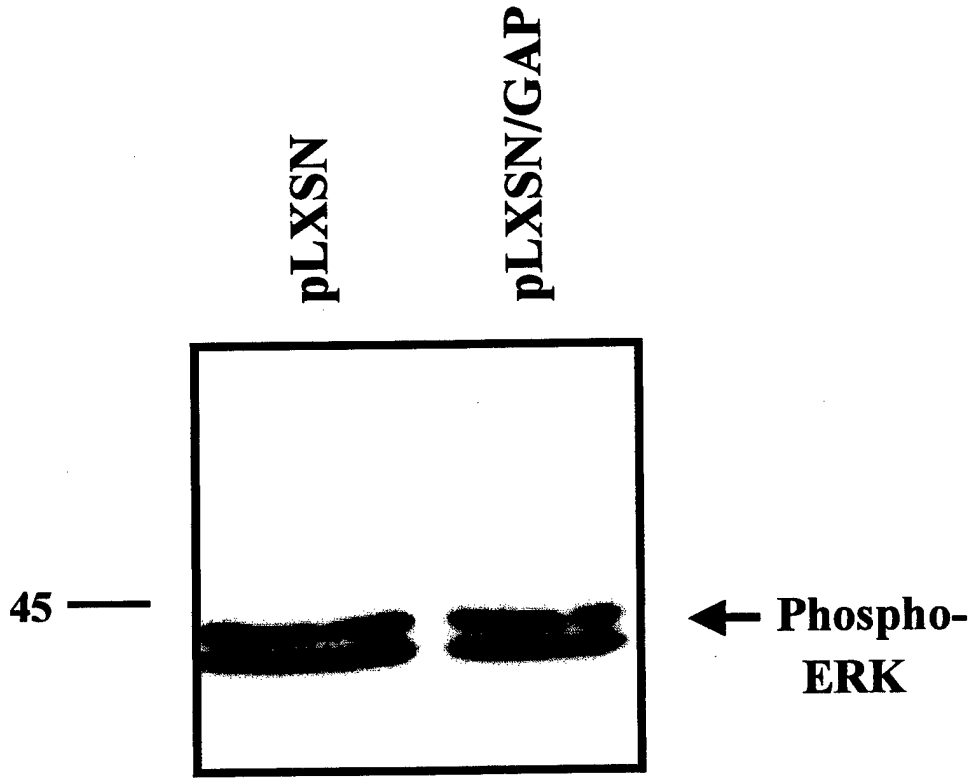


Fig.6: Northern Blot Analysis. Total RNA (25 ug) from ST88-14 cells stably transfected with empty vector (pLXSN) or p120GAP (pLXSN/GAP) was size fractionated over formaldehyde-agarose gels and hybridized to ^{32}-P labeled cDNA probes for human protein tyrosine phosphatase-δ (PTP-δ), rat tropomyosin-α, rat transgelin, and an oligonucleotide probe for the 18S ribosomal RNA as a control for loading.

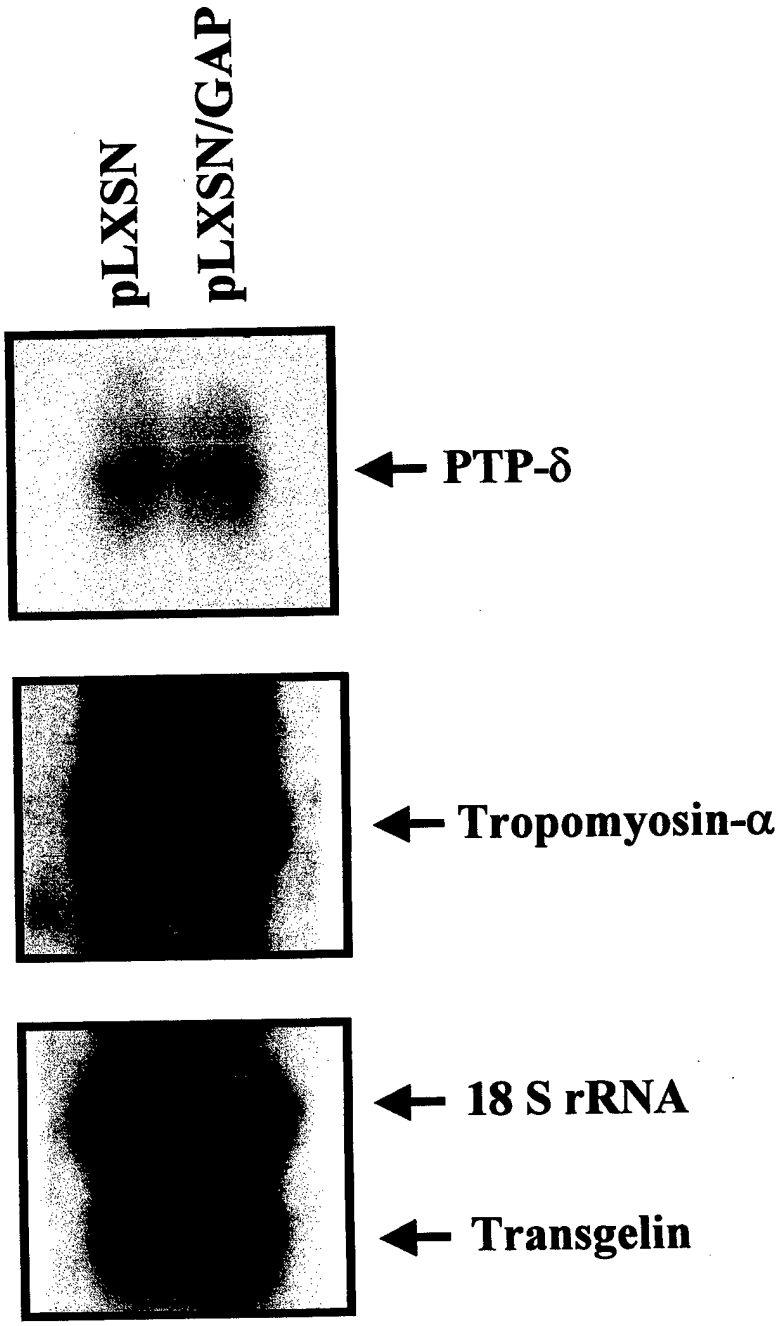
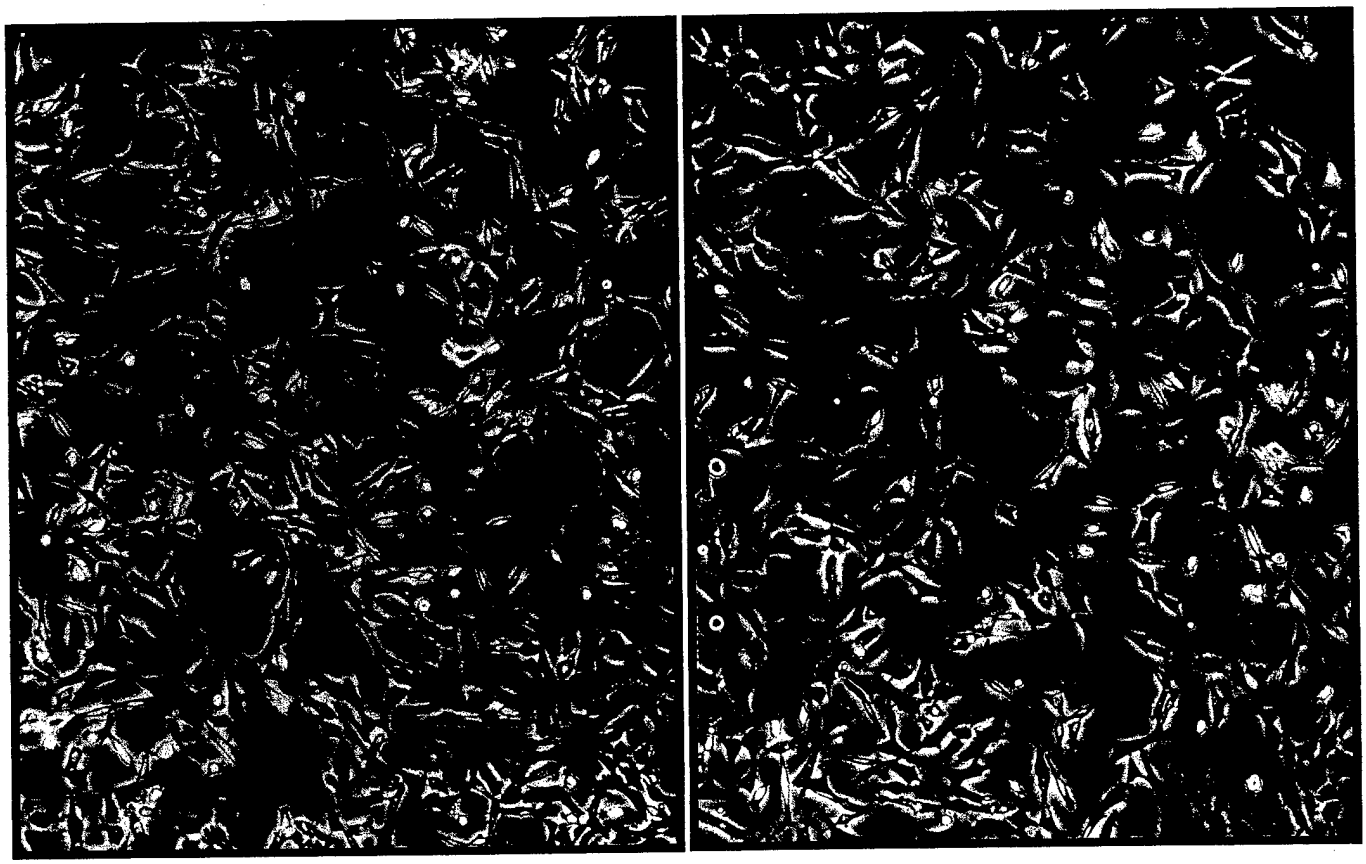


Fig.7: Morphology of Cells Stably Expressing p120 C-GAP. Mass populations of ST88-14 cells stably expressing p120GAP truncated at the N-terminus (C-GAP) were generated by retroviral infection. Cells were infected with virus generated with the pZIP-NeoSV(x)1 retroviral vector, where expression of the inserted C-GAP gene is regulated by the Moloney Long Terminal Repeat promoter, and selected with 400 ug/ml G418. Shown on the left are empty vector (pZIP) infected cells and on the right are pZIP-C-GAP expressing cells. Note that the cells expressing C-GAP are slightly larger and flatter than the control vector-only cells.



pZIP **pZIP / C-GAP**

Fig 8: Total ERK Expression in ST88-14/C-GAP Cell Lysates. Equal amounts of protein lysates (30 ug) were prepared from ST88-14 cells stably expressing C-GAP, separated over a 12 % SDS-PAGE gel, transferred to Immobilion-P, blotted with anti-total ERK serum (Santa Cruz) at 1:1,000 and detected with HRP-conjugated secondary antibody with ECL. Shown are lysates from empty vector infected cells (pZIP) and those expressing C-GAP (pZIP/C-GAP) grown in low (0.1%) or normal (10%) serum.

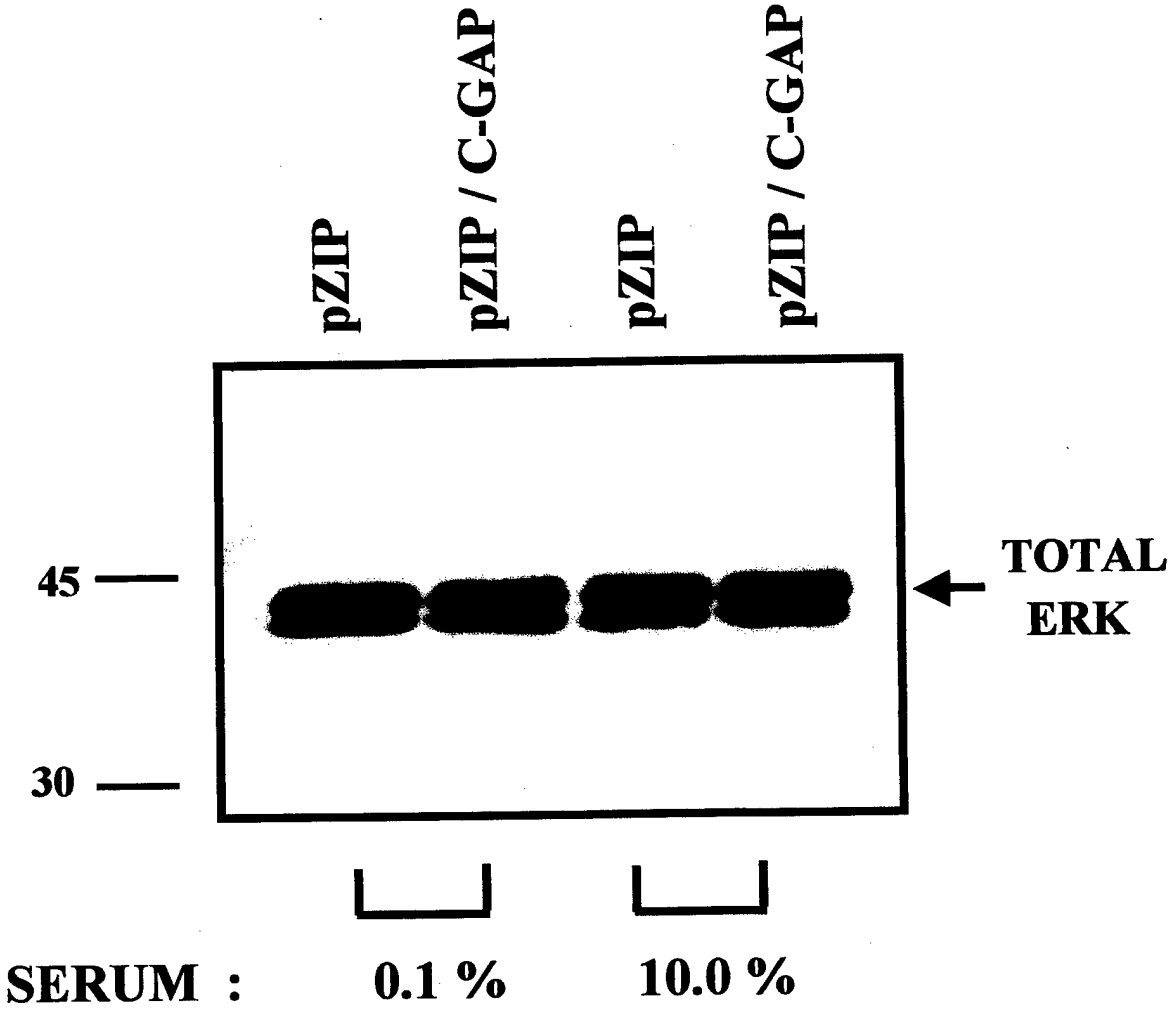


Fig.9: Ras-GTP Levels are Decreased in Cells Overexpressing C-GAP. Equal amounts of protein lysates (500 ug) prepared from ST88-14 cells stably expressing C-GAP or vector only were mixed with 5 ug Raf-1 RBD-agarose (Upstate), washed, separated over a 12 % SDS-PAGE gel, transferred to Immobilion-P, blotted with anti-pan-Ras serum (Oncogene Research Products) at 2.5 ug/ml and detected with HRP-conjugated secondary antibody with ECL. Shown are Ras-GTP levels from lysates of empty vector infected cells (pZIP) and those expressing C-GAP (pZIP/C-GAP) grown in 0.1% or 10% serum 24h .

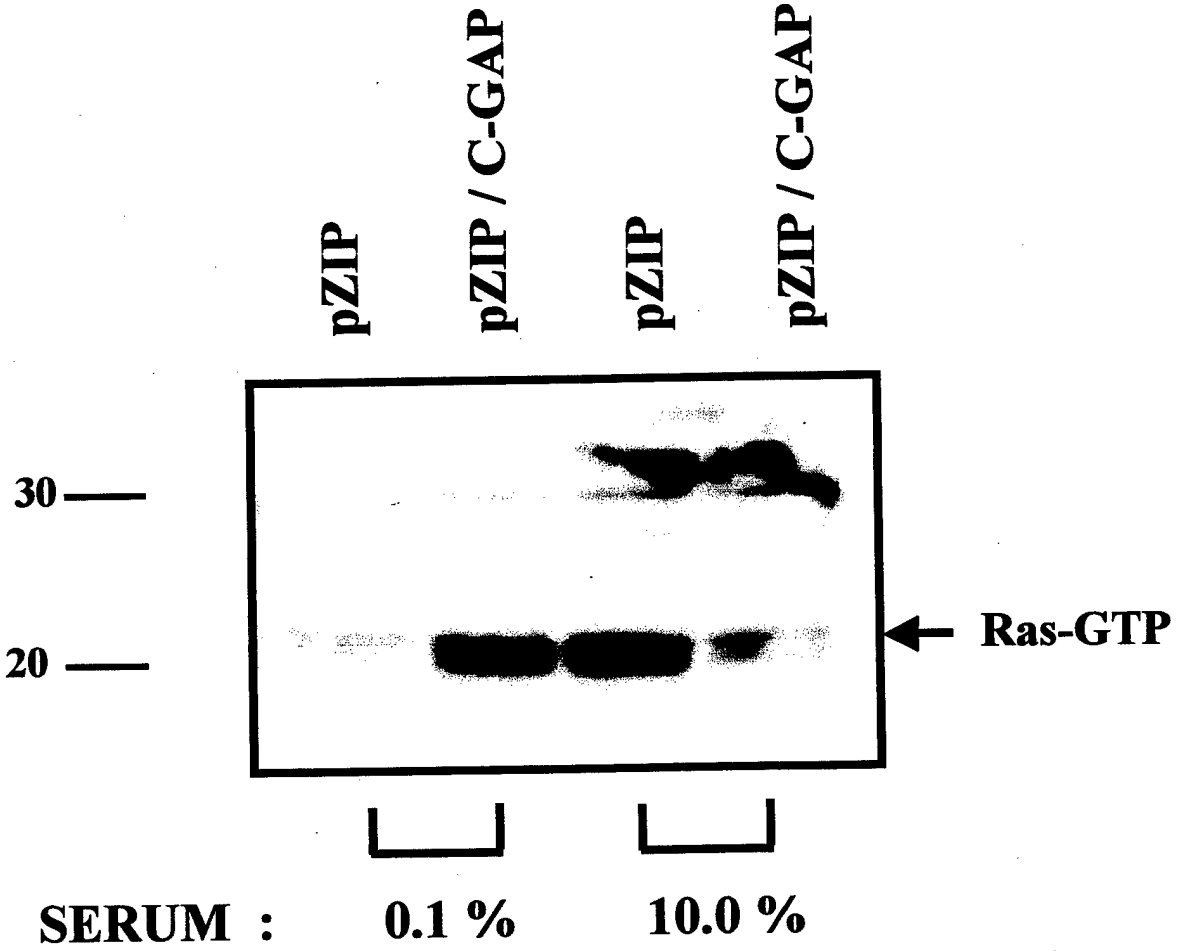


Fig.10: Phospho-ERK Levels are Not Altered in Cells Overexpressing C-GAP. Equal amounts of protein lysates (30 ug) were prepared from ST88-14 cells stably expressing GAP, separated over a 12% SDS-PAGE gel, transferred to Immobilion-P, blotted with anti-phospho-p44/42 MAPK serum (Cell Signaling) at 1:1,000 overnight and detected with HRP-conjugated secondary antibody with ECL. Shown are lysates from empty vector infected cells (pZIP) and those expressing C-GAP (pZIP/C-GAP) grown in 0.1% or 10% serum 24h.