

Evaluation of the Small Molecular Weight G-Protein Ras in the Interaction of Growth Hormone and Epidermal Growth Factor Signaling

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Abstract

It has been shown that epidermal growth factor (EGF) and growth hormone (GH) send opposing messages in 3T3-F442A fibroblast cells; EGF sends the signal to divide whereas GH sends the signal to differentiate. Although these two extracellular messengers stimulate distinct cellular outcomes, when cells expressing both receptor types bind these molecules, EGF mediated mitogenesis is decreased by 50%. In this study, we sought to develop an approach to separate and analyze the actions of EGF and GH in these fibroblasts in order to determine the mechanisms responsible for the GH-associated decrease in EGF-mediated mitogenesis. To accomplish this goal we treated cells with a TAT dominant negative (dn) Ras protein. We chose to use dn Ras because Ras is thought to be an important molecule for GH signaling. After treatment with the TAT dn Ras protein, the cells were treated with GH and EGF, and we found that GH activation of ERK1, ERK 2, and STAT 5 was attenuated by the TAT dn Ras protein. ERK 1 and ERK 2 are part of a group of mitogen activated protein kinases (MAPKs) that send signals to other effector molecules that can result in altered receptor function, inhibition or activation of transcription, and apoptosis. STAT 5 is a transcription factor that, upon activation, can either halt or promote transcription by traveling to the nucleus of the cell and binding to a promoter sequence on the DNA. Furthermore, we found that the dn Ras did not appear to affect EGF activation of ERK1 and ERK 2 suggesting alternative pathways may be involved. This preliminary data provides the foundation for further study into the actions of Ras and its effects upon STAT 5 phosphorylation/activation, a previously undocumented finding. Further investigations of GH mediated STAT 5 activation through Ras should prove instrumental in explaining the actions by which GH attenuates EGF-induced mitogenesis.



Introduction

At the cellular level, cancer is simply a mass of cells (often starting from a single cell) that divide without bounds and migrate throughout the body. At a more systemic level, cancer can shut down whole organ systems and cause the death of the entire organism, while creating intense havoc for the individual, their family, their friends, and their caretakers. At a more macroscopic level, cancer is responsible for 555,000 deaths per year in the United States alone, making it the second leading cause of death in America (1). In addition to loss of life, cancer costs us a total of \$156.7 billion every year in direct medical costs, costs due to loss of productivity, and indirect mortality costs (1). Regressing slightly to the subcellular level, cancer can be caused by intra- and extracellular signals gone awry. The signals and pathways that dictate whether a cell will divide, migrate, differentiate, die, or become transformed (cancerous) are called signal cascades and the process is referred to as signal transduction. The Bertics' lab studies a myriad of signaling pathways and how they relate to asthma, septic shock, and cancer. The focus of this research project is the pathway traveled to uncontrolled cell division, which causes tumors (cancer).

Although there are many different pathways that induce mitogenesis, and several that prevent it, the focus of our research is on two powerful pathways and two powerful hormones that we believe to be key elements in the cellular processes that lead to cancer and may hold the keys to unlock the corridor that holds the cure. The first signaling event that we are looking at begins with a small, polypeptide hormone called epidermal growth factor (EGF). When this extracellular messenger binds to its receptor (epidermal growth factor receptor [EGFR]), it sends a message to the cell. Cell type, time period within the cell cycle, and age of the cell are determinants of which type of message will be sent. In the cells that we are using, 3T3-F442A murine fibroblasts, EGF induces mitogenesis (2).

The second signaling event that we are studying begins with growth hormone (GH)—and like EGF—can also signal a myriad of different events; however, in our F442A cells, GH is known

to induce differentiation(2). Both EGF and GH are known to activate a pathway that uses a molecule known as Ras (Figure 1). The portions of this pathway that we focused on in our study are EGF binding to EGFR or GH binding to growth hormone receptor (GHR), the activation of Ras, and the activation of 44kDa and 42 kDa mitogen-activated protein kinases (MAPKs) called ERK 1 and ERK 2, respectively. Upon phosphorylation, ERK 1 and ERK 2 send messages to other effector molecules that may result in alteration of enzyme activity, alteration of receptor function, or activation/inhibition of transcription(3).

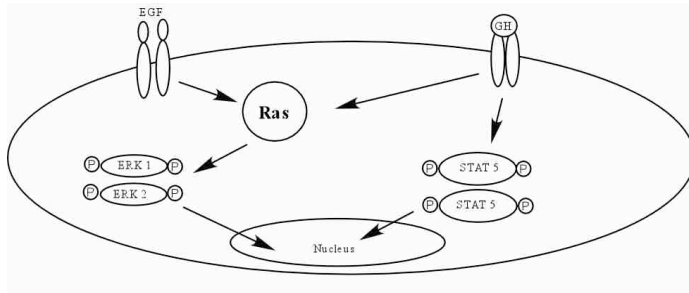


Figure 1. GH and EGF Signaling Pathways. Both GH and EGF are known to activate ERK 1 and ERK 2 using a Ras dependent pathway. Whereas, GH, but not EGF, is known to phosphorylate STAT 5.

Another signaling pathway, called the Jak/STAT pathway, is activated by GH but not by EGF(3). The portions of the Jak/STAT cascade that we focused upon are GH binding to GHR and the phosphorylation and dimerization of a transcription factor called STAT 5. Once phosphorylated, this 90 kDa transcription factor travels to the nucleus and affects transcription(4) (Figure 1). In the case of GH-mediated action, EGF-induced transcription is blocked(3). In the type of cell that we used, F442A murine fibroblasts, both EGFR and GHR are present. When high levels of both EGF and GH bind to their respective receptors, EGF-induced mitogenesis is decreased by 50%; however, the mechanisms behind this action are unclear (2).

This brings us to our hypothesis, which is to find the specific GH signaling pathway that is essential for attenuating EGF-stimulated mitogenesis. In order to test our hypothesis, we developed an approach to separate the actions of GH from those of EGF. Our approach was to develop a TAT dn Ras protein. A TAT protein is derived from an HIV sequence that is able to diffuse through the cell membrane and into the cell. This TAT sequence is then bound to the dn Ras sequence and the dominant negative protein can now be delivered into the cell and inhibit the function of the normal Ras protein (5).

Materials and Methods

Cell Culture and Reagents

Murine 3T3-F442A cells were maintained at a subconfluent level in DMEM supplemented with 10% calf serum at 37°C and

5% CO₂. Cells were suspended/passaged using a 0.1% trypsin solution and plated at 25,000 cells/60 mm plate in DMEM/10% calf serum and then cultured under these conditions for 48-72 hours. The cells were then serum-starved for 24-48 hours in DMEM/0.1% BSA to induce quiescence and synchronize a majority of the cell population into the G₀ portion of the cell cycle prior to treatment.

Treatment of Cells

Cells treatments included: 0.31 μM (5 μL), 0.94 μM (15 μL), 1.9 μM (30 μL), or 3.1 μM (50 μL) of a TAT dn Ras protein for 30 minutes; control buffer (Hank's Balanced Salt Solution), 10 nM of GH, 10 nM of EGF, 10 nM of GH and 10 nM EGF, or 100 nM of phorbol 12-myristate 13-acetate (PMA) for 10 minutes. PMA is non-receptor mediated activator of the MAPKs and is included as a positive control.

Immunoblotting

The cells were lysed and loaded onto acrylamide gels and electrophoresed. The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes, blocked with a 5% milk solution and probed with either anti-active MAPK, anti-MAPK, anti-Phospho-STAT5 (Tyr 694) or anti-whole STAT5 antibody. The membranes were then treated with a 1:7500 solution of goat anti-rabbit IgG with horseradish peroxidase for one hour. And antibody binding was detected using chemiluminescence.

Results

Dose Dependent Response of TAT dn Ras

To find the optimal dosage of the TAT dn Ras protein, we executed dose-response experiments using either untreated cells or cells treated with 10nM of EGF in the presence of various doses of TAT dn Ras protein [0.31 μM (5 μL), .94 μM (15 μL), or 1.9 μM (30 μL)]. We then immunoblotted for anti-active MAPK antibodies to detect phosphorylated ERK 1 and ERK 2. EGF stimulated ERK 1 and ERK 2 phosphorylation; however, none of the doses of the TAT dn Ras protein attenuated EGF-induced ERK phosphorylation (Figure 2). This suggested that either the concentration of TAT dn Ras that was used was too low, the TAT dn Ras was inactive, or that Ras was not the only effector molecule used by EGF to activate ERK 1 and 2.

Effects of the TAT dn Ras Protein on GH Activation of MAPK

In the dose dependent experiment, the TAT dn Ras protein did not attenuate EGF-mediated ERK phosphorylation. In order to rule out an inactive dn Ras protein, we performed the following experiment: cells were treated with Control, GH, EGF, PMA in the presence or absence of 3.1 μM of TAT dn Ras. In light of the fact that both EGF and PMA are known to activate ERK 1 and ERK 2 through different mechanisms, PMA was used as the positive control. We then immunoblotted using an anti-active

phospho-ERK antibody. The GH lane yielded 42 and 44 kDa bands whereas the GH/dn Ras lane did not show any bands. The EGF alone and EGF/dn Ras did not exhibit any changes (Figure 3). This suggested that the TAT dn Ras protein was active and that it was able to abrogate GH-mediated ERK 1/2 phosphorylation. In addition, it reinforced the previous experiment that suggested that Ras was not the only effector molecule used by EGF to activate ERK 1 and ERK 2.

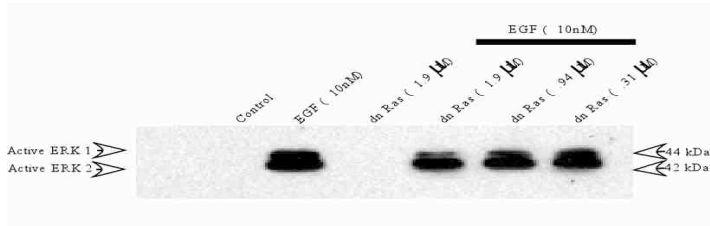


Figure 2. Effects of various doses of TAT dn Ras on EGF-mediated ERK 1/2 activation. Serum-starved 3T3-F442A fibroblasts were treated with or without EGF in the presence of different doses of TAT dn-Ras at the concentrations listed above. The treatment times are listed in materials and methods section. Cells were then lysed and subject to immunoblot analysis using an anti-active MAPK antibody.

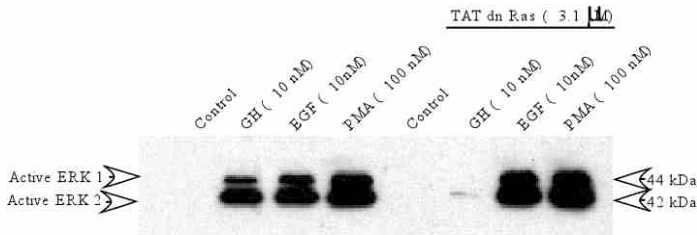


Figure 3. Effect of TAT dn Ras on GH and EGF-Mediated ERK 1/2 Activation. Serum-starved 3T3-F442A fibroblasts were treated with or without TAT dn Ras in the presence or absence of GH, EGF, PMA, at the concentrations listed above as described in the Materials and Methods section. After treatment, cells were lysed and subjected to immunoblot analysis using an anti-active MAPK antibody.

STAT Activation by the Ras Pathway

In order to investigate the STAT pathway, we used GH since it is known that GH treatment results in the phosphorylation of STAT5 and it is thought that GH specifically uses the Jak/STAT pathway to do so. To show that GH may phosphorylate STAT 5 using another pathway—a Ras dependent pathway—we treated the cells with the TAT dn Ras protein for thirty minutes. Then, those cells were either untreated, treated with 10 nM of GH, 10 nM of EGF, or 100nM of PMA for ten minutes. Other cells were treated GH, EGF, or PMA for ten minutes and did not receive TAT dn Ras treatment. Next, we did an immunoblot using an anti-phospho STAT 5 (Tyr 694) antibody. In the anti-phospho STAT 5 probe, a band appeared in the GH-treated lane at the 90 kDa level. The band was much lighter in the dn Ras/GH treated lane. No other bands were seen (Figure 4a). This indicated that GH activation of STAT 5 is dependent upon Ras.

In order to ensure that reduced STAT 5 phosphorylation in the GH/TAT dn Ras lane was not due to lack of the STAT 5 protein, the membrane that was used for the anti-phospho STAT 5 immunoblot was stripped and reprobbed for whole STAT. Bands were seen in every lane at the 90 kDa level at approximately the same intensity (Figure 4b) indicating an equal loading of proteins.

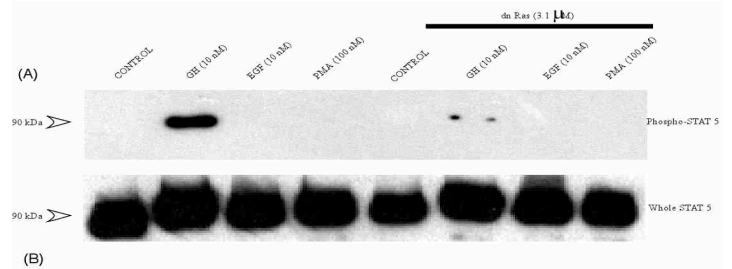


Figure 4. Effect of dn Ras on GH-mediated STAT 5 phosphorylation. (A) Serum-starved cells were treated with either GH, EGF, or PMA, with or without dn Ras at the concentrations listed above. Cells were then subject to immunoblot analysis using an anti-active phospho STAT 5 (Tyr 694) antibody. This experiment was performed two separate times with comparable results. (B) The membrane was subsequently stripped of all bound antibodies and reprobbed with a whole STAT5 antibody to confirm equal loading between lanes.

Discussion

In order to investigate two of the known GH pathways—the Ras and STAT paths—we elected to use a TAT dn Ras protein. By using this protein, we hoped to isolate the steps in the pathway that GH uses to abrogate EGF-mediated mitogenesis. We were successful in purifying the TAT dn Ras; and therefore, we needed determine the level of activity and find an appropriate dosage to block Ras-dependent signaling. EGF is known to powerfully activate the MAPKs, ERK 1 and ERK 2, which have been shown to be sensitive to Ras dependent pathways, so this is where we began experimenting. Surprisingly, the TAT dn Ras protein did not substantially inhibit the phosphorylation of either ERK 1 or ERK 2 at any of the concentrations used (Figure 2). One explanation for this observation was that a much higher concentration of TAT dn Ras is necessary for blocking MAPK phosphorylation. Another possible explanation was that our TAT dn Ras protein was not functional. The third possibility was that Ras is not the only player in the EGF-stimulated path to ERK 1/2 phosphorylation.

The next step we took was to investigate whether our TAT dn Ras was working properly. Because GH is known to activate ERK 1 and ERK 2, we assessed whether the activation of GH, EGF, and PMA were differentially sensitive to the addition of TAT dn Ras. In addition to adding GH and PMA to this experiment, we also used a higher concentration of dn Ras than was used in the previous experiments, i.e. we increased the concentration by 67%. As seen in Figure 3, GH-induced activation of ERK 1 and ERK 2 was completely blocked by the addition of TAT dn Ras. This suggested that our TAT dn Ras

was in fact working properly. Furthermore, it suggested that EGF-induced ERK phosphorylation may not solely involve Ras, e.g. ERK activation pathways may bifurcate somewhere upstream of Ras. Finally, this experiment showed that the GHR is dependent upon Ras for activation of the ERKs.

GH is also known to phosphorylate STAT 5, which is part of the Jak/STAT pathway. However, it is suspected that there is more than one site on STAT 5 that must be phosphorylated for its activation. When the phospho-STAT 5 immunoblot was done, we found that the GH lane showed an immunoreactive band at 90 kDa (Figure 4a). This was expected since GH has been previously shown to activate STAT 5 (3). However, the GH/dn Ras lane showed a greatly diminished phospho-STAT 5 band, which was somewhat unexpected because Ras has not been previously implicated in the activation of STAT 5. This suggests several different possibilities. The first is that when Ras is not functional, the dead-end complexes formed in response to GHR activation lead to molecular crowding, thereby attenuating JAK/STAT access and activation. Another possibility is that Ras is a critical component in the coupling of GHR to STAT 5 phosphorylation. Also, TAT dn Ras does not appear cytotoxic because EGF-stimulated pathways are unaffected. Because there are molecules that are downstream of Ras, it is not known which molecule is directly responsible for the phosphorylation of STAT 5; thus, further study in this area is needed. It would be interesting to obtain both TAT dn MEKK and TAT dn ERK proteins, which are both downstream of Ras, to narrow the possibilities.

Next, we treated another set of F442A fibroblasts with TAT dn Ras, GH, EGF, and PMA and analyzed for phospho-STAT 5 content by immunoblotting. The conditions used in the previous experiment were also used in this experiment, except that one extra lane was added. In the extra lane, we added 10nM of GH and 50 uL of TAT green fluorescent protein (GFP). This was added as a control to ensure that the anti-phosphorylative action of the TAT dn Ras was due to the dn Ras rather than the TAT because the GFP should not affect STAT 5 phosphorylation. As expected, all lanes that were present in the first phospho-STAT 5 experiment remained the same and the GH/GFP lane exhibited a 90 kDa band at a similar intensity to the GH only lane (Data not shown). This suggests that the actions of the TAT dn Ras protein are can be attributed to the dn Ras rather than the TAT portion.

In order to ensure that the other lanes in the STAT 5 immunoblot were not empty due to a loading error, we stripped the blot and reprobed for whole STAT 5. If any of the lanes appeared to be lacking, this would mean that there were not enough cells or protein added to that lane. As Figure 4b shows, the whole STAT content was equivalent in each lane. This blot reinforces the validity of the phospho STAT 5 blot not only in the loading aspect but also in the phosphorylation aspect. The 90 kDa band in lane 2, the GH lane, is noticeably higher than the rest of the

bands. Because STAT 5 is slightly more massive when it is phosphorylated, it migrates downward less than the unphosphorylated version.

In the future, we will use a flow cytometer to quantify DNA synthesis. This measure will indicate the amount of DNA per cell, which will reveal whether or not our cells are proliferating. In addition, we will also analyze cyclin D expression in our cells by immunoblotting. Cyclin D is a protein that is required for cell cycle progression. When cells are not advancing through the cell cycle, cyclin D is not expressed. Therefore, this test should also indicate whether our cells are dividing or resting.

In summary, these data have led us to two sets of conclusions. First, dn Ras may serve as a useful tool in dissecting the pathway of GH and EGF. This is important because it may help to explain how GH attenuates EGF induced mitogenesis. Once the inhibitory mechanisms of GH are understood, GH could serve as a useful tool in developing therapies that inhibit uncontrolled cell division like we see in cancer. The second conclusion that we can make from our data is that Ras may not only be essential for GH activation of ERK 1 and ERK 2 but that Ras may be linked to the activation STAT 5. STAT 5 is a transcription factor that regulates which genes are transcribed and which are not. Some genes that STAT 5 may be responsible for blocking are those that are responsible for leading the cell through the cell cycle and causing cell division. If indeed Ras is a key component in the activation of STAT 5, we have come one step closer to finding a way to manipulate the transcription of potentially harmful genes, such as the ones that are active cancerous cells.

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