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Activated c-Met signals through PI3K with dramatic effects on cytoskeletal functions in small cell lung cancer

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Abstract

Small cell lung cancer (SCLC) is an aggressive illness with early metastases. There are several receptor tyrosine kinases (RTKs) overexpressed in SCLC, including c-Met. c-Met contains an external semaphorin-like domain, a cytoplasmic juxtamembrane domain, tyrosine kinase domain and multiple tyrosines that bind to adapter molecules. We have previously reported that c-Met is abundantly expressed in the NCI-H69 SCLC cell line and now have determined the downstream effects of stimulating c-Met via its ligand hepatocyte growth factor (HGF). Utilizing unique phospho-specific antibodies generated against various tyrosines of c-Met, we show that Y1003 (binding site for c-Cbl and a negative regulatory site), Y1313 (binding site for PI3K), Y1230/Y1234/Y1235 (autophosphorylation site), Y1349 (binding site for Grb2), Y1365 (important in cell morphogenesis) are phosphorylated in response to HGF (40 ng/ml, 7.5 min) in H69 cells. Since multiple biological and biochemical effects are transduced through the PI3K pathway, we determine the role of PI3K in the c-Met/HGF stimulation pathway. We initially determined that by inhibiting PI3K with LY294002 (50μM over 72 hours), there was at least a 55% decrease in viability of H69 cells. Since H69 SCLC cells form clusters in cell culture, we determined the effects of HGF and LY294002 on cell motility of the clusters by time-lapse video microscopy. In response to HGF, SCLC moved much faster and formed more clusters, and this was inhibited by LY294002. Finally, we determined the downstream signal transduction of HGF stimulation of c-Met with and without inhibition of c-Met (with geldanamycin, an anisamycin antibiotic that inhibits c-Met in SCLC) or PI3K (with LY294002). We show that association of c-Met with PI3K and GAB2 is diminished by inhibiting c-Met. In summary, activation of the c-Met pathway targets the PI3K pathway in SCLC and this may be an important therapeutic target.

Keywords: signal transduction in lung cancer • PI3-K • cell motility • c-Met/HGF • geldanamycin

Introduction

Small cell lung cancer (SCLC) is an aggressive illness with a poor prognosis, and unfortunately the biological processes that underlie the disease are currently not well defined. Recently, tyrosine kinase receptors have been implicated in the etiology of multiple tumor types and they may be important therapeutic targets [1, 2]. We have previously reported that the receptor tyrosine kinase c-Kit is important in SCLC and that it can be specifically targeted by the tyrosine kinase inhibitor STI 571

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[3]. This finding has been applied clinically with the use of STI 571 in a phase II clinical trial for patients with previously untreated SCLC and patients with relapsed drug-sensitive disease [4]. Most recently, we have identified that c-Met is overexpressed and functional in SCLC [1].

c-Met is an RTK, which stimulates the invasive growth of carcinoma cells, is tumorigenic, potentially mutated and overexpressed in many solid tumors [5]. The c-Met receptor is a disulfide-linked α-β heterodimer with a molecular weight of 190 kDa [6]. The 140 kDa β-chain spans the membrane and possesses cytoplasmic tyrosine kinase activity, and can be detected in its precursor form at 170 kDa. The overexpression of c-Met and activated mutations can lead to carcinogenesis in multiple tumors. The best-characterized mutations have been identified in hereditary renal cell carcinoma and are mainly in the tyrosine kinase domain [7]. When activated by autophosphorylation, c-Met can associate with and activate multiple signal transducing intermediates such as Grb2, the p85 subunit of PI3-kinase, Stat-3, and Gab1 [8]. Overexpression of c-Met has been shown in SCLC and non-small cell lung cancer (NSCLC) cells. We have recently shown that c-Met is overexpressed and activated in response to the binding of its ligand hepatocyte growth factor (HGF) in SCLC cells [1]. In particular, NCI-H69 SCLC cells show robust expression of c-Met and undergo dramatic biochemical and biological changes in response to HGF on cell motility and migration. Herein, we studied phosphatidylinositol 3-kinase (PI3K) specifically as an important part of c-Met downstream signaling.

The PI3K pathway controls a number of cellular processes including cytoskeletal organization, cell growth and survival [9-11]. PI3K is activated by growth factors, oncogenes, chemokines, cell surface receptors and integrins [12]. Activation of PI3K has been shown to be critical for signaling pathways and the sub-groups of the PI3K family are involved in migration, adhesion, motility and proliferation in various cell types [13]. It has been demonstrated that constitutive PI3K activity in SCLC regulates proliferation, anchorage-independent growth and apoptosis [12]. Inhibitors of PI3K (for example, wortmannin and LY294002) are currently being evaluated in combination with chemotherapy in cell lines and animal models [14].

The aims of this study were to investigate the role of c-Met/HGF signaling through PI3K in SCLC and to identify signal transduction mediators involved in c-Met/HGF signaling. We have particularly studied the PI3K pathway's role in mediating cell motility and migration of SCLC cells.

Materials and methods

Cell line and cell culture

The NCI-H69 (hereafter referred to as H69) SCLC cell line was obtained from the American Type Culture Collection (Rockville, MD), and maintained in RPMI 1640 media (Cellgro), 10% (v/v) fetal calf serum (FCS) and 1% (v/v) penicillin-streptomycin [1]. Cells were incubated at 37ºC in a humidified, 5% CO_2 in air atmosphere. For stimulation studies with HGF, cells were deprived of growth factors by incubation in serum-free medium containing 0.5% BSA (Sigma, St. Louis, MO) for 16 hours. H69 cells have been previously shown to have robust expression of c-Met [1]. For kinetic analysis experiments, H69 were stimulated with or without HGF (40 ng/ml, [1]). H69 cells were incubated for 24 h and 48 h with 40 ng/ml of HGF, and in the presence or absence of the PI3K inhibitor LY294002 at 50 μM [15]. H69 cells were also incubated with 100 ng/ml HGF and with or without LY294002 (50 μM) to observe the impact of HGF and PI3K on the rate of cellular coalescence (visualized by time-lapse video microscopy). We have previously determined that this concentration is optimal for studying cell motility in SCLC.

Antibodies and chemicals

The anti-phosphotyrosine monoclonal antibody, mAb #4G10, was obtained from UBI (Lake Placid, NY). Rabbit polyconal antibodies against c-Met (C-12) (Santa Cruz, CA) Akt, Akt (pSer 473), PI3K-p85 and Gab2 (Cell Signaling Technology, Inc, Beverly, MA) and the anti-β-Actin monoclonal antibody (Sigma, St. Louis, MO) were used as per the manufacturer's directions. We used polyclonal phosphorylation site-specific antibodies (Biosource International Camarillo, CA) to tyrosine1003 (PTP-S, c-Cbl binding site), tyrosine1313 (PI3K binding site), tyrosine1230/1234/1235 (autophosphorylation

site), tyrosine 1349 (Grb2 binding site), and tyrosine 1365 (important in cell morphogenesis) in c-Met. Phosphatidylinositol-3 kinase inhibitor LY294002 was obtained from Cell Signaling Technology (Beverly, MA). Geldanamycin was obtained from the National Cancer Institute and was used to determine the effects of c-Met expression in H69 cells with and without HGF. A concentration of 1 μM was used for the biochemistry assays, as defined previously [1].

Cell viability assay

NCI-H69 cells (1×10^6/ml) were cultured in medium with or without 40 ng/ml HGF. Viable cells were counted by trypan blue dye exclusion every 24 h up to 72 h. Each data point was repeated in triplicate.

Preparation of cell lysates and Immunoblotting

Cells were lysed in lysis buffer **(**containing 20 mM Tris, pH 8.0; 150 mM NaCl, 10% glycerol, 1% NP40, 0.42% NaF, 1 mM PMSF, 1 mM Na_3VO_4, 5 μg/ml aprotonin, 5 μg/ml leupeptin**)** as described previously [1]. Cell lysates were separated by 7.5% SDS-PAGE under reducing conditions, electrophoretically transferred to an immobilization membrane (Schleicher & Schuell, Keene, NH), and immunoblotted using the enhanced chemiluminescense technique (NEN Life Science Products, Boston, MA). Immunoprecipitations and immunoblotting were also performed according to standard procedures [1].

Cell Motility Assay with Time Lapse Video Microscopy

Serum-starved H69 cells were placed in uncoated plastic tissue culture plates (35×10 mm plates, Becton-Dickinson Labware), and treated with or without 100 ng/ml of HGF and with or without 50 μM LY294002. The plate was incubated at 37ºC in a 5% CO_2 in air, fully humidified atmosphere. Cells were examined by TLVM using an Olympus IX70 inverted microscope, Omega temperature controlled device, DVC1310 digital video camera, and QED Camera with Standalone 145 software. Images were recorded for a period of 4 hours for each set of experiments performed, with a digital video image saved every minute. The digital video images were then processed with an Apple computer containing a G4 microprocessor, and the cell movement or morphological changes were analyzed using the NIH Image Analysis program. For movement analysis, the position of cell centroid was measured every 5 min by outlining each cell or cluster on every 5th frame. The use of x/y coordinates and the NIH Image Analysis Program allowed us to map cells and clusters of cells. The time taken for cells or clusters to form a bigger cluster was noted. All these values were tabulated as was the distance that each cell or cluster traveled. The distance that the cell or cluster centroid transversed for each minute was calculated to determine the speed of movement.

Results

Dose-response and kinetic study of HGF stimulation on tyrosine of several proteins and on serine 473 of Akt in SCLC

Previously we have shown that the expression of c-Met was elevated in the H69 cell line as compared to other SCLC cell lines [1, 16]. We have now studied tyrosine phosphorylation of various proteins and serine phosphorylation of Akt at position 473 in response to different concentrations of HGF (0-100 ng/ml, Fig 1A) and at different time points (0-1 hr) following HGF (40 ng/ml, Fig 1B) using H69 as SCLC model. Figure 1A (upper panel) demonstrates that there was increased tyrosine phosphorylation of multiple proteins (60-170 kDa) when H69 was stimulated with different concentrations of HGF for 7.5 min. Increased tyrosine phosphorylation was visualized at 170 kDa, 140 kDa, 120 kDa, 90 kDa, 70 kDa, and 60 kDa in response to HGF. Stimulation of H69 with 25 ng/ml of HGF for 7.5 min significantly increased tyrosine phosphorylation. The levels of expression of phospho-S473-Akt and β-Actin were investigated by immunoblot analysis. Increased Akt phosphorylation was observed with equal quantities of protein loaded (Fig 1A, middle and bottom panel).

In a kinetic analysis, maximal tyrosine phosphorylation occurred within 1-7.5 min of stimulation with HGF (40 ng/ml) (Fig 1B, upper panel). The tyrosine phosphorylation signal decreased with progression of time, eventually returning to basal lev-

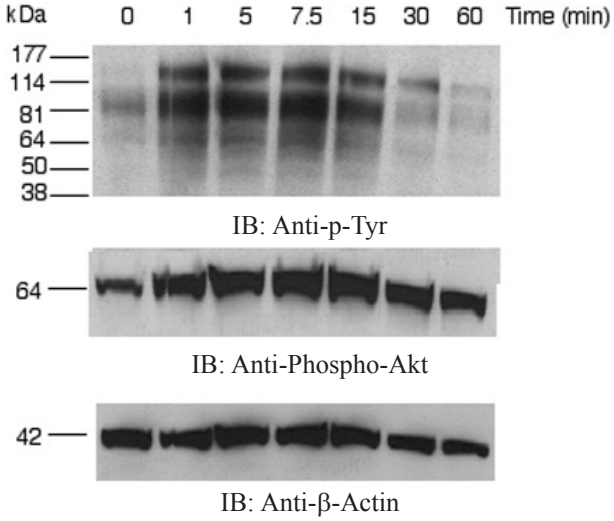


Fig. 1A Dose response HGF stimulation on tyrosine phosphorylation in the H69 SCLC cell line. Cell lysates for the H69 cell line were processed as described in "Materials and Methods". Lysates were applied on a 7.5% SDS-PAGE gel, transferred to an Immobilon-P membrane, and probed with the anti-phospho tyrosine, anti-phospho Akt, and β–actin. Tyrosine phosphorylated proteins were detected in cellular lysates by immunoblotting using an anti-phospho tyrosine antibody [upper panel], anti-phospho-Akt antibody [middle panel]. The β-actin control of the same membrane shows the similar protein loading [bottom panel].

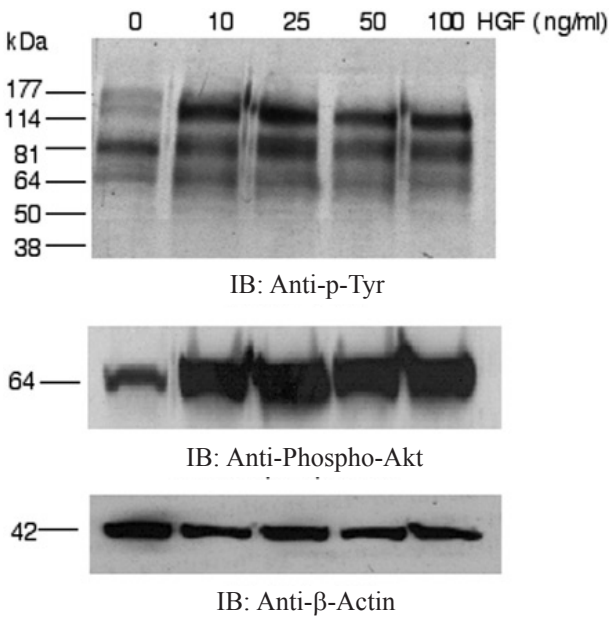


Fig. 1B Kinetics study of HGF stimulation on tyrosine phosphorylation in the H69 SCLC cell line. Tyrosine phosphorylated proteins were detected in cellular lysates by immunoblotting using an anti-phospho tyrosine antibody [upper panel], anti-phospho-Akt antibody [middle panel]. The β-actin control of the same membrane shows the similar protein loading [bottom panel].

els. In order to confirm the Akt activation and demonstrate equal protein loading, membranes were stripped and immunoblotted with phospho-S473-Akt (Figure 1B, middle panel) and β-Actin (Figure 1B, bottom panel).

Phosphorylation of specific tyrosine residues of c-Met by HGF stimulation in SCLC

Previously, we have shown strong tyrosine phosphorylation of c-Met in response to HGF in H69 SCLC cells. We have obtained specific antibodies against important intracytoplasmic tyrosines of c-Met to determine which tyrosines are activated in response to HGF in SCLC. Various phospho-specific c-Met antibodies [anti-phospho-tyrosine (pY)1003, pY1313, pY1349, pY1365 and pY1230/1234/1235-c-Met] were utilized to assess the functionality of the HGF/c-Met signaling pathway (structure shown in Figure 2A). Lysates of H69 cells with or without HGF (40 ng/ml, 7.5 min) stimulation were immunoprecipitated (IP) with c-Met antibody and the membranes were immunoblotted (IB) with the particular phosphospecific c-Met and c-Met antibodies. The results demonstrated that HGF stimulation of the c-Met receptor tyrosine kinase in H69 increased the phosphorylation of tyrosines1003 (PTP-S, c-Cbl binding site), 1313 (PI3K binding site), 1230/1234/1235 (major ligand-induced autophosphorylation site), 1349 (SHC, Src and Gab1 binding site) and 1365 (site known to inhibit cell morphogenesis) in response to HGF . The amount of c-Met immunoprecipitated was the same with or without HGF (Fig. 2B).

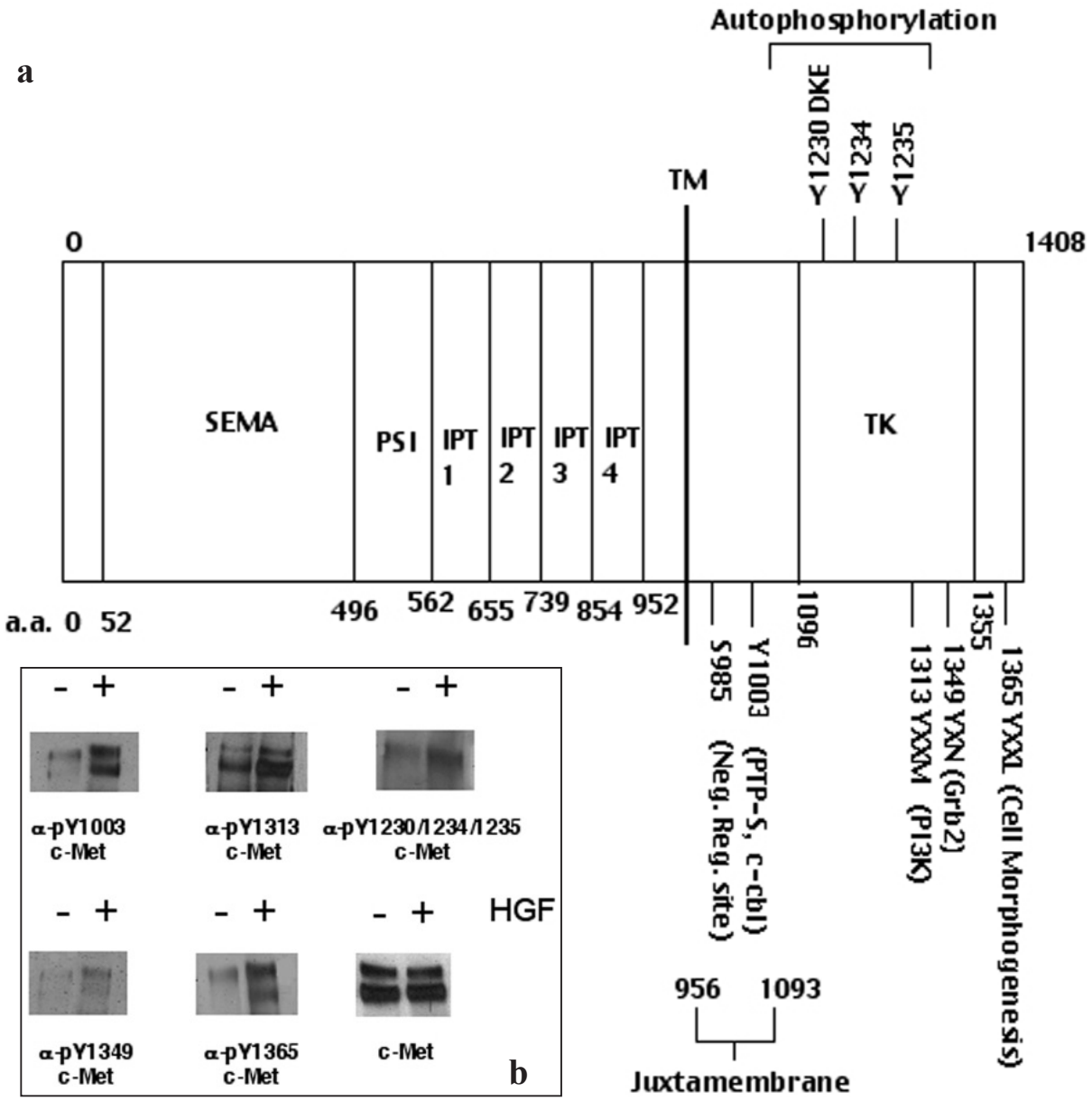


Fig. 2 a) Predicted functional domains of c-Met. Depicted here are the Sema-domain (semaphorin-like), the PSI-domain (found in plexins, semaphorins, and integrins), the IPT-repeat-domains (found in Ig-like regions, plexins, and transcription factors), and the TK domain (tyrosine kinase located, intracellularly). The various amino acid residues with regulatory functions for the c-Met/HGF pathway are illustrated here.

b) Phosphorylation of various tyrosine residues of c-Met in response to HGF in SCLC. Cell lysates for the H69 cell line were processed as described in "Materials and Methods". Lysates were treated with or without HGF (40 ng/ml; 7.5 min), were immunoprecipitated with anti c-Met. The whole cell lysate and immunoprecipitations were immunoblotted with anti phospho tyrosine pY1003, pY1313, 1230/1234/1235, pY1349 and pY1365 of c-Met and anti c-Met antibody.

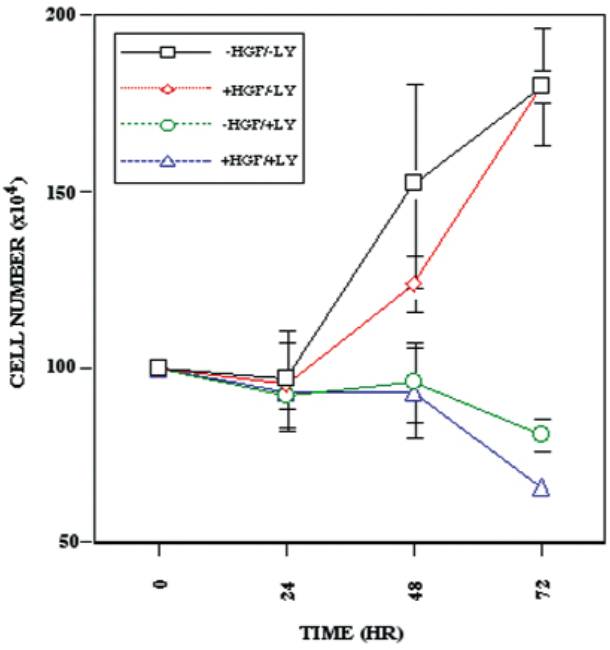


Fig. 3 SCLC viability in response to HGF and PI3K inhibition. Viability assay in response to (-) or (+) HGF, and (-) or (+) LY294002 at each time point - 24 hr, 48 hr, and 72 hr were performed to observe the effects on cell growth and viability. H69 cells were cultured in serum containing media and stimulated with (-) or (+) HGF (40 ng/ml), and (-) or (+) LY294002 (50 μM). Viable cells were counted with trypan blue dye exclusion method. ata were plotted over a period of 72 hr. Error *bars*, SEMs of four separate experiments.

Biological functions associated with PI3K

Since one of the key tyrosines phosphorylated was the PI3K binding site, we attempted to determine whether PI3K has an important role in SCLC.

Cell viability

We tested the hypothesis that the PI3K inhibitor LY294002 would inhibit HGF-induced H69 cell growth and viability. H69 were either left untreated (-HGF) or treated with HGF (40 ng/ml) in the absence or presence of LY 294002 (50 μM). Incubation was performed over 72 hours in serum containing media. As seen in figure 3, in absence of LY294002, the cell number increased 1.8 fold in 72 hours irrespective of the presence of HGF. The proliferation of cells in the absence or presence of HGF was similar over 72 hours. In the presence of LY294002, the rate of proliferation decreased significantly over the 72 hour time frame irrespective of the presence of HGF. For both cases, the highest rate of decrease was between 48 and 72 hours. Therefore, the PI3K inhibitor LY294002 appears to block the proliferation of H69 cells.

Cell motility

Time-lapse video microscopy analysis of cellular morphology, motility and clustering of serum starved H69 cells without or with HGF (100 ng/ml) was performed for 4 hours in the presence or absence of LY294002 (50 μM). The cell motility of the SCLC cells was analyzed by determining the morphology, speed, and formation of clusters as shown in Figures 4A-4C. The cells were usually clustered and in response to HGF, they became further conglomerated (Figure 4A). Data analysis was performed with the NIH-Image Analysis Program that allowed us to achieve frame-to-frame analysis of positions of each cell or cellular cluster in a temporal fashion. The speed was calculated for the centroid of each cluster. We investigated the effect of HGF on the resultant speed of cellular motility/migration for H69 treated with or without HGF (100 ng/ml) in the presence or absence of LY294002. It was observed that in the absence of LY294002, the speed of the clusters was higher (mean = 0.26 μm/min) (with or without HGF) as compared to the cells in the presence of LY 294002 (mean = 0.091 μm/min) (figure 4B). It was also observed that in the absence of LY 294002, the average speed for the cells treated with HGF (speed = 0.35 μm/min) was much higher than that without HGF (speed = 0.17 μm/min). There was no significant difference in the speeds when cells treated with or without HGF were also treated with the PI3K inhibitor LY 294002 (Figure 4B). However, as compared to no treatment, the LY 294002 treated cells had dramatically decreased speed.

We also analyzed the ability of cellular clusters to coalesce to form larger clusters as a function of cellular motility/migration (Fig. 4C). This is because SCLC tends to travel in clusters in vivo, and HGF can induce larger cluster formation. As

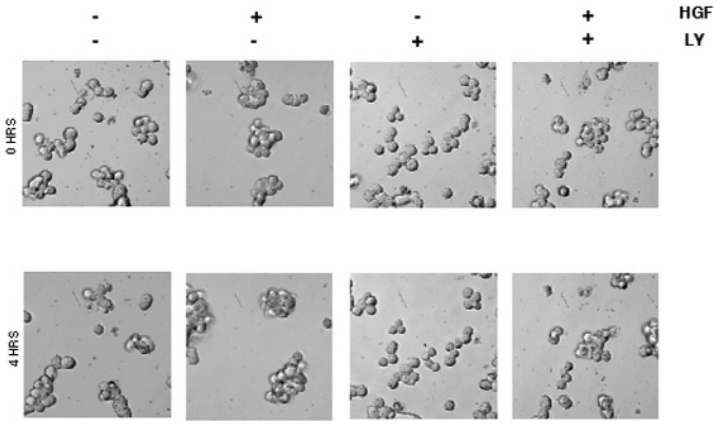
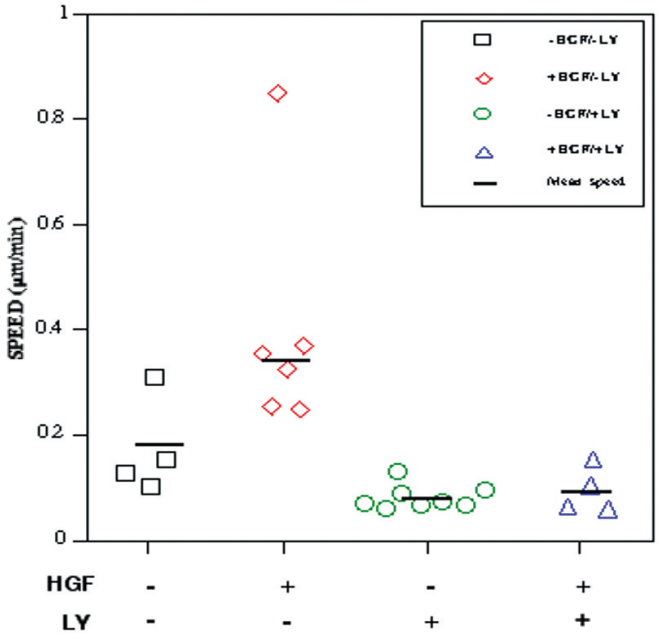
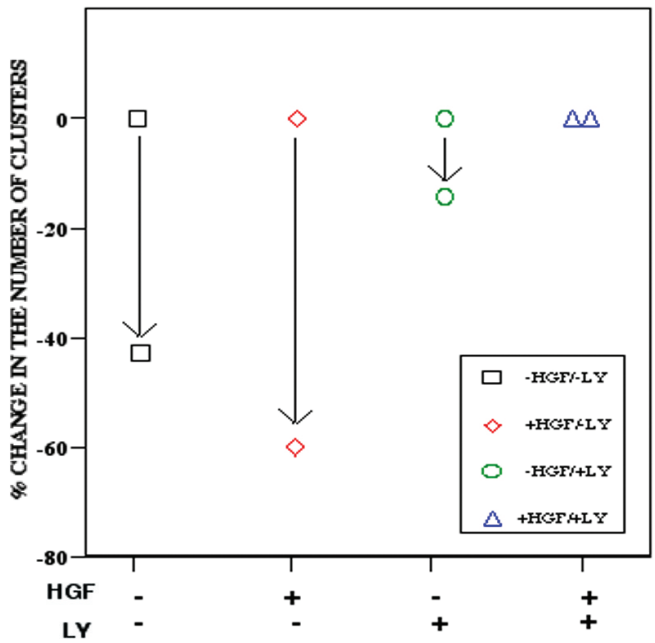


Fig. 4 A SCLC morphology in response to HGF and PI3K inhibition. H69 cells were starved overnight and treated with or without HGF (100 ng/ml) and with or without LY294002 (50 μM). Shown are representative pictures before and after 4 hours with the conditions.



B SCLC cellular speed in response to HGF and PI3K inhibition. Time-lapse video microscopy analysis in terms of speed of H69 cells in response to (-) or (+) HGF (100 ng/ml), and (-) or (+) LY294002 (50 μM). Serum-starved H69 cells were visualized by time-lapse video microscopy and recorded for 4 hours with or without HGF and with or without LY294002. These images were then analyzed by NIH image analysis and each cell/cluster in every frame was traced every 5 minutes. The position of the cell centroid was measured and the corresponding X and Y axes values were noted. The distance transversed by each cell/cluster was calculated using these values and from this the speed was determined for each cell/cluster. These data are represented for each set of experiment and the corresponding average speed has also been shown.



C SCLC cellular clusters in response to HGF and PI3K inhibition. Time-lapse video microscopy analysis in terms of the percentage change of total number of clusters of H69 cells in response to (-) or (+) HGF, and (-) or (+) LY294002 (50 μM) over a period of 4 hours. Serum-starved H69 cells were visualized by time-lapse video microscopy and recorded for 4 hours with or without HGF and with or without LY294002 (50 μM). These images were then analyzed by NIH image analysis and each cell/cluster in every frame was traced every 5 minutes. The coming together of the smaller clusters to form larger ones was followed over a period of 4 hours. These data have been plotted as the % change in the total number of clusters formed for each of the 4 sets of experiments.

shown in Fig. 4C HGF stimulated formation of larger clusters through combination of cellular clusters of smaller sizes in the culture medium. Also, the number of cluster formation over a 4 hour period was reduced in the presence of LY294002. In these experiments, all the cellular clusters were accounted for, and traced every minute over a period of 4 hours. No cells/clusters moved in or out of the time-lapse video microscopy frame during the 4 hour examination period.

Taken together, the results demonstrate that in the absence of PI3K inhibitor, HGF increased the speed of cellular migrational movement. Further, LY294002 significantly abolished the stimulatory effects of HGF on cellular migrational speed and decreased the basal speed of SCLC cells in the absence of HGF.

Inhibition of c-Met association with PI3K and Gab2 with geldanamycin

c-Met immunoprecipitations

Whole cell lysate (WCL) of H69 cells treated without or with HGF (7.5 min, 40 ng/ml) in the absence or presence of geldanamycin (1 μM) were immunoprecipitated (IP) with an anti-c-Met antibody and the WCLs and IPs were immunoblotted with anti-phosphotyrosine (Fig. 5, upper panel). Results showed that geldanamycin inhibited HGF induced tyrosine phosphorylation in WCL proteins as well as immunoprecipitation with the c-Met antibody (Fig. 5, upper panel). Geldanamycin, a c-Met inhibitor, inhibited HGF induced tyrosine phosphorylation of c-Met at 1230/1234/1235 (Fig. 5, second panel). We subsequently investigated the interaction of c-Met with PI3K in response to HGF in this particular cell line. The data revealed that there was increased interaction of c-Met with PI3K in response to HGF, whereas the association was significantly reduced following treatment with geldanamycin (Fig. 5, third panel). There was constitutive association of c-Met with Gab2 with or without HGF. With geldanamycin treatment, there was decreased association of c-Met with Gab2 (Fig. 5, fourth panel). Finally, data revealed that there was a decreased amount of c-Met following geldanamycin treatment (fig. 5A, bottom panel).

PI3K immunoprecipitations

Whole cell lysate (WCL) of H69 cells treated without or with HGF (7.5 min, 40 ng/ml) in the absence or presence of geldanamycin (1 μM) were immunoprecipitated (IP) with anti-PI3K (p85) antibody and the WCLs and IPs were immunoblotted with anti-phosphotyrosine and anti-PI3K-p85 respectively (Fig. 6). Results demonstrated that geldanamycin inhibited HGF induced tyrosine phosphorylation in WCL proteins as well as immunoprecipitation with the PI3K antibody (Fig. 6, upper panel). The amount of PI3Kp85 immunoprecipitation was the same irrespective of HGF or geldanamycin in both cases of WCL and IPs (Fig. 6, bottom panel).

Inhibition of PI3K association with Gab2 with LY294002 treatment

We next investigated the role of PI3K in mediating the downstream signaling and physiological responses in SCLC with the use of specific PI3K inhibitor, LY 294002 (Fig. 7). Whole cell lysate (WCL) of H69 cells treated without or with HGF (7.5 min, 40 ng/ml) in the absence or presence of LY2940029 (50 μM) were immunoprecipitated (IP) with anti-PI3K (p85) antibody and the WCLs and IPs were immunoblotted with anti-phosphotyrosine, anti-Gab2 and anti-PI3K-p85 respectively (Fig. 7). A significant decrease in baseline as well as HGF-induced protein tyrosine phosphorylation was observed following LY294002 treatment (Fig. 7, upper panel). The results were confirmed further with the use of immunoprecipitation with anti-PI3K antibody under the same experimental conditions. Subsequent immunoblotting with anti-phosphotyrosine revealed that PI3K is involved in the HGF/c-Met signaling axis with an impressive level of HGF-induced phosphorylation. Further this HGF-induced PI3K phosphorylation was specifically abrogated by LY294002 (figure 6, top panel). This phosphorylation of tyrosine may be in Gab2 protein. Under the same experimental conditions, it was observed that there was a decreased amount of Gab2 in response to LY294002 treatment (middle panel). There was increased association of Gab2 and PI3K with HGF stimulation and this was decreased in response to LY294002. Data showed that the amount of PI3K immunoprecipitation was

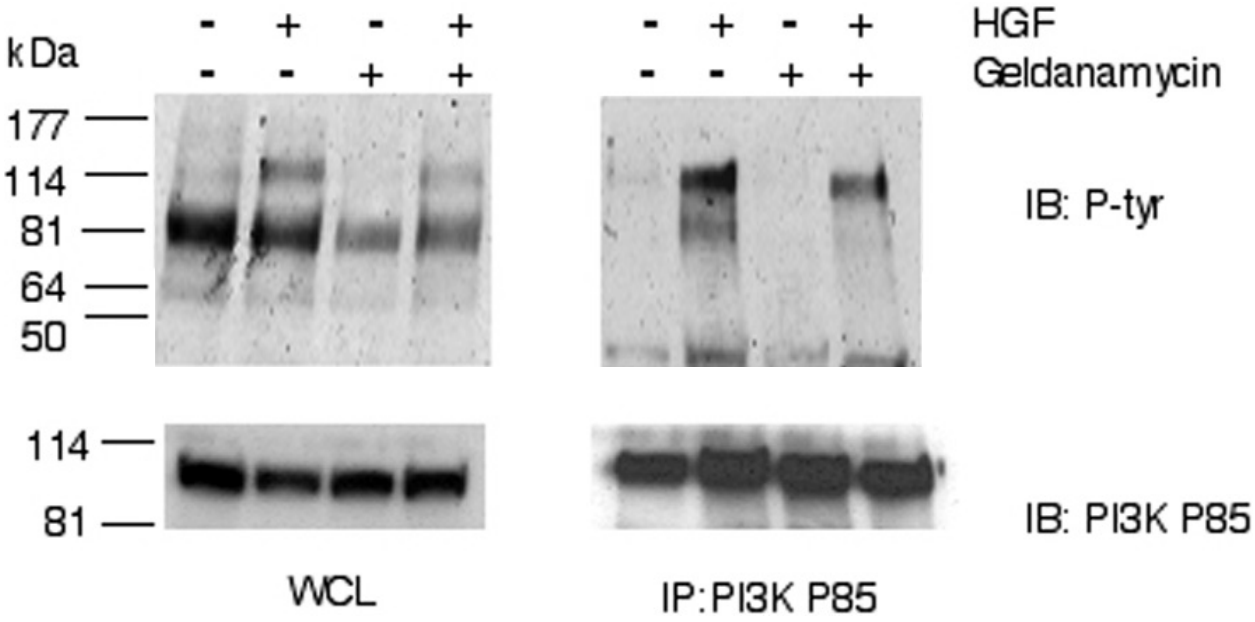


Fig. 5 Tyrosine phosphorylation of PI3K in response to HGF and effect of geldanamycin treatment in H69 cells. H69 cells treated with or without HGF (40 ng/ml; 7.5 min) and with or without geldanamycin (1 μM) were immunoprecipitated with anti phospho PI3K p85. Whole cell lysate and immunoprecipitations were processed and immunoblotted with anti phospho-tyrosine antibody [upper panel]. The same membrane was stripped and immunoblotted with anti PI3K p85.

same irrespective of the presence of HGF or of LY294002 in the WCL and IPs under the same experimental conditions (Fig. 6, bottom panel).

Discussion

Small cell lung cancer is a unique clinical illness characterized by early metastasis. Even though the molecular biology of SCLC has been well understood, the cellular biology has to be defined further [2]. We report the importance of c-Met/HGF signaling through the PI3K pathway in SCLC. In these studies, it has been shown that c-Met is phosphorylated on several unique tyrosines in response to HGF in SCLC. Also, c-Met activation leads to increased cell motility as reflected through the speed and formation of cellular clusters. c-Met acts downstream through PI3K since inhibition of this pathway through LY294002 leads to decreased cell motility. Inhibition of c-Met with geldanamycin also leads to decreased association of c-Met with PI3K and Gab2.

c-Met is a unique RTK that is expressed in lung cancer cells and stimulated by its ligand HGF, expressed on stromal cells**.** Overexpression of c-Met has been detected in SCLC and NSCLC cells. In a study by Olivero *et al*. [17], c-Met was found to be increased 2-10 fold in 25% of NSCLC tumor tissue as compared to adjacent normal tissue. In a further study by Ichimura *et al.* [18], the expression of c-Met was determined by immunoblotting and shown to be positive in 11/11 NSCLC cell lines studied, and demonstrated in 34/47 (72%) adenocarcinoma and 20/52 (38.5%) squamous cell carcinoma tumor tissues. c-Met protein expression tended to correlate with higher pathological tumor stage and a worse clinical outcome. c-Met expression, at both mRNA and protein levels, have been described in 22/25 SCLC cell lines and nude mouse xenografts [19]. We have now determined that c-Met is phosphorylated on specific amino acid sites for further defining its function in lung cancer.

In this study, we have shown that c-Met is specifically phosphorylated on several tyrosine amino acids in response to HGF in SCLC. The tyrosine sites 1230, 1234, and 1235 are located in the

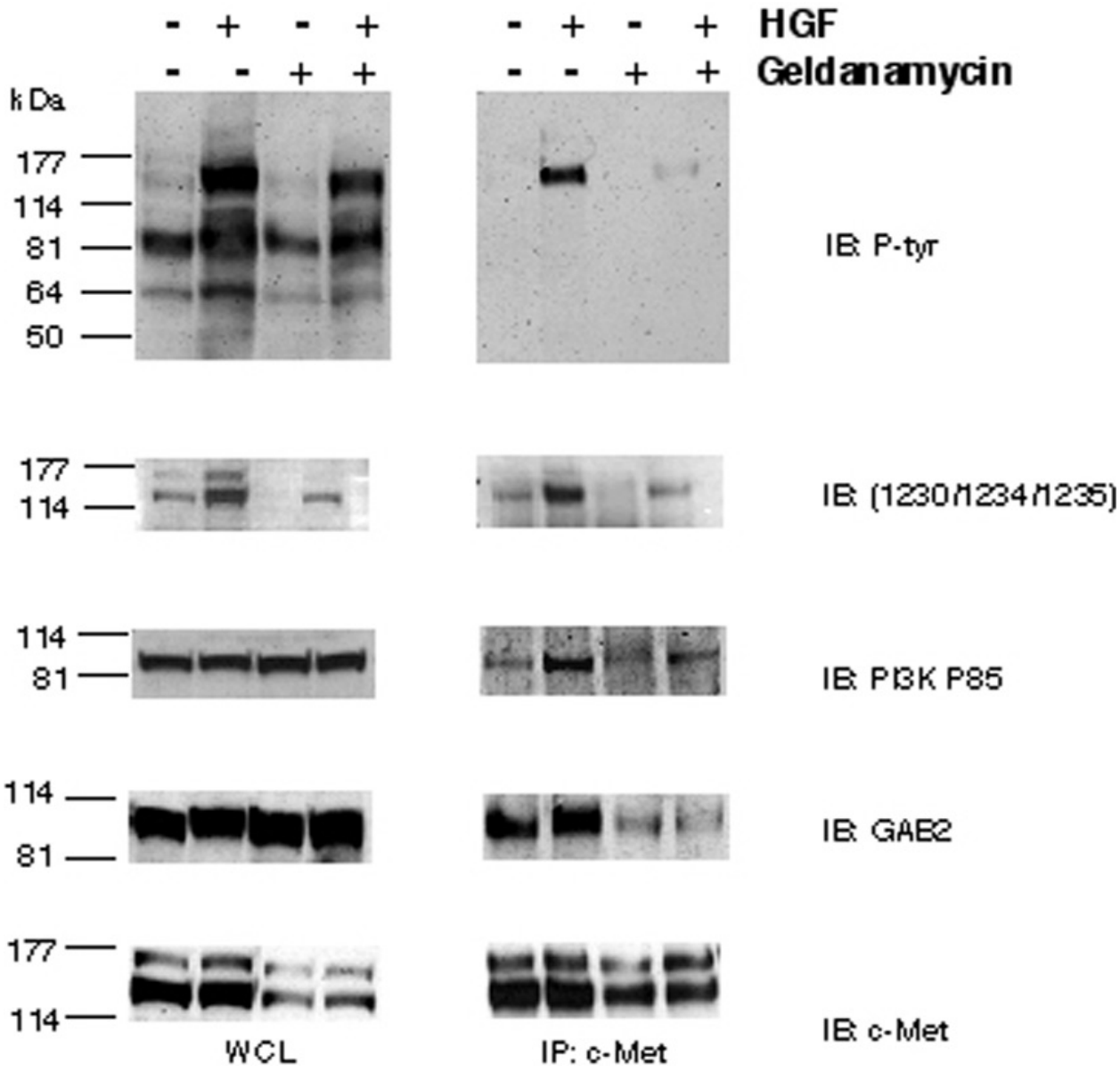


Fig. 6 Tyrosine phosphorylation of PI3K in response to HGF and effect of geldanamycin treatment in H69 cells. H69 cells treated with or without HGF (40 ng/ml; 7.5 min) and with or without geldanamycin (1 μM) were immunoprecipitated with anti phospho c-Met. Whole cell lysate and immunoprecipitations were immunoblotted with anti phospho- tyrosine [upper panel], thereafter with anti pY1230/ pY1234/pY1235 [second panel], anti PI3K p85 [third panel], anti GAB2 [fourth panel], and anti c-Met [bottom panel].

tyrosine kinase domain and are important in autocatalytic function of c-Met. Tyrosine 1003 is important in binding to proteins such as c-Cbl and is central to the juxtamembrane (JM) domain of c-Met. JM domains of RTKs have been shown to be key regulators of catalytic functions [20]. As examples, in the PDGFR-β RTK, autophosphorylation of JM tyrosines 579 and 581 is required for stimulation of RTK activity [21]. Conversion of these tyrosines to phenylalanine inhibits receptor activation, while substitution of a valine residue, N-terminal to the regulatory tyrosines, results in constitutive receptor activation *in vitro* and *in vivo* [22]. Also, phosphorylation of JM tyrosines creates a binding site for

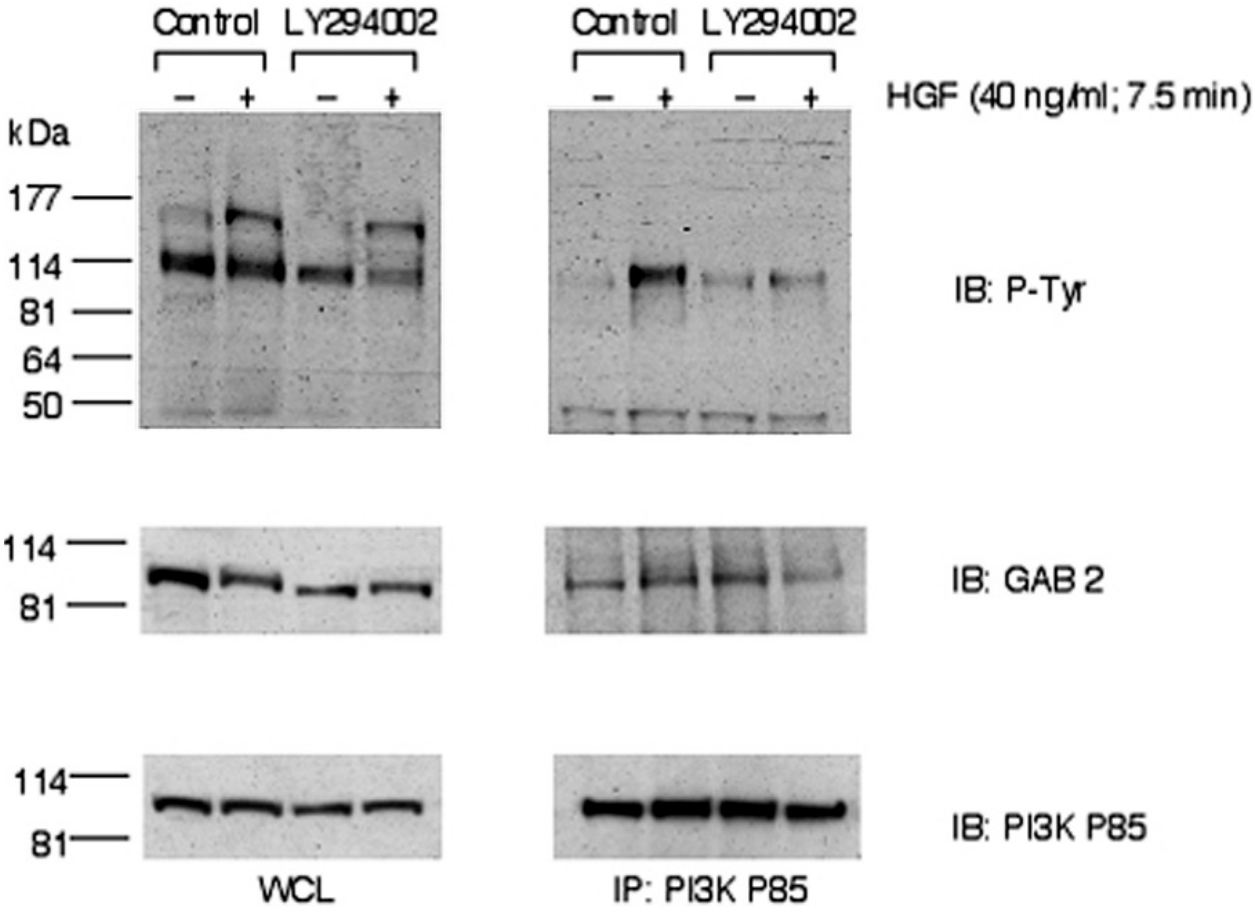


Fig. 7 Association of PI3K with GAB2 in response to HGF and the effect of PI3K inhibition in H69 cells. H69 cells were treated with or without HGF (40 ng/ml; 7.5 min) and with or without LY294002 (50 μM). Whole cell lysates under the above conditions were immunoprecipitated with anti-PI3K p85. The whole cell lysates and immunoprecipitations were immunoblotted with anti p-Tyr [upper panel], then with anti-GAB2 [middle panel], and then with anti-PI3K p85 as the control [bottom panel].

the Src SH2 domain, resulting in Src recruitment to the receptor. As another example, in acute myeloid leukemias, 20% of tumors have internal tandem duplications of another RTK, Flt3. The mutation in Flt3 creates an in-frame insertion of variable length in the JM region, leading to ligand-independent kinase activity and oncogenic activation [23-25]. These data suggest that JM regions repress kinase activity, and that JM mutations that relieve this inhibition can lead to oncogenesis. Thus, in the future, it would be important to determine the implications of this in SCLC and determine if there are specific mutations that are activating or inhibitory.

Tyrosine at position 1313 is also phosphorylated in response to HGF in SCLC. This tyrosine is important in binding to PI3K (with the YXXM motif). We show here that inhibiting the PI3K pathway leads to decreased cell growth and viability of SCLC. HGF stimulation of cell motility and migration is also inhibited with LY294002. The mechanism whereby HGF stimulation of c-Met leads to increased motility, migration and invasion is not well understood. c-Met stimulation promotes cell movement, causes epithelial cells to disperse ("scatter") and endothelial cells to migrate, and promotes chemotaxis. Evidence exists that enhanced cell motility and invasion may be an important consequence of c-Met signaling. For example, mutant mice nullizygous for Met show that muscles in the limb, diaphragm and tip of the tongue, which normally originate from migrating dermomyotome cells, fail to develop [26].

Cell motility has been shown to be tightly controlled by the lipid kinase PI3K and p21GTPases including Ras, Rac and Rho [27, 28]. PI3K appears to be an important molecule in HGF-induced mito-, moto- and morpho-genesis, since inhibition of PI3K by wortmannin leads to decreased branching formation on collagen matrix and chemotaxis of renal cells [29, 30]. Microinjection of activated H-Ras stimulates cell spreading and actin reorganization, whereas inhibition of endogenous Ras abolishes spreading, actin reorganization and scattering [31-33]. In A549 NSCLC cells, HGF stimulation increases Ras-GTP by 50% [34]. c-Met has been previously shown to bind Gab1. The Gab family (Gab1 and Gab2) contains a pleckstrin homology (PH) domain and potential binding sites for SH2 and SH3 domains [35]. Gab family members act as scaffolding adapters that bind various signal molecules, including protein-tyrosine Shp-2 and PI3K, in response to stimulation through cytokine and growth factor receptors [36]. Gab proteins are involved in Flt3 signaling to mediate downstream activation of Shp-2, PI3K pathways and possibly the Ras/Raf/Mapk pathway [37]. Overexpression of Gab2 in Jurkat cells or antigen specific T cell hybridomas resulted in the inhibition of NFAT activation, IL-2 production and tyrosine phosphorylation [38]. It has been reported that Gab2 acts as the principal activator of PI3K in response to epsilon RI activation thereby regulating the PI3K pathway *in vivo* [36]. Our finding that c-Met binds to Gab2 in SCLC is unique and not reported previously.

Geldanamycin has been described as an inhibitor of HSP90 function [39]. Hsp90 functions as a chaperone, to assist proteins to acquire mature conformation, and has been shown to associate with tyrosine kinases such as v-Src, LCK, and p210BCR-ABL. In a recent study by An, et al [40], geldanamycin altered the association of Hsp90 with p210BCR-ABL, thereby leading to the degradation of the cellular tyrosine kinase. We have shown that there was indeed a decreased amount of c-Met in response to geldanamycin treatment [1]. However, this may not be related to direct association with Hsp90, since cross-immunoprecipitation did not reveal any direct interaction between these two molecules (data not shown). Possibilities are that c-Met either may not be synthesized as fast as non-geldanamycin treated cells, or that c-Met is being degraded via ubiquitination in response to geldanamycin. We have previously shown that Hsp70 levels are increased by geldanamycin [1]. Hsp70 has been implicated in apoptosis events as related to reactive oxygen species [41]. It has been recently reported that extracellular HSP70 act as a chaperone and possesses cytokine activity. HSP70 binds with plasma membrane, elicits a rapid intracellular Ca^{2+} flux, activates NF-κB and upregulates the expression of pro-inflammatory cytokines such as IFN-γ, TNF-α and IL-6 [42]. There are several analogs of geldanamycin available, and the effects on SCLC along with HGF/c-Met should be further explored. Finally, an inhibitor specifically targeting HGF/c-Met would be quite useful for SCLC studies.

In summary, we show that HGF stimulation of c-Met leads to specific phosphorylation of several tyrosines. The PI3K pathway is crucially targeted via c-Met stimulation, and it would now be useful to test small molecule inhibitors of c-Met in the PI3K pathway and eventually in clinical trials.

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