An 11-Amino Acid Sequence from c-met Initiates Epithelial Chemotaxis via Phosphatidylinositol 3-Kinase and Phospholipase C*

Melanie P. Derman, Janet Y. Chen, Katherine C. Spokes, Zhou Songyang, and Lloyd G. Cantley

From the Division of Nephrology, Beth Israel Hospital, Harvard Medical School, Boston, Massachusetts 02215

Interaction of hepatocyte growth factor with its high affinity receptor c-met initiates a cascade of intracellular events leading to epithelial motility. An 11-amino acid sequence from the c-met receptor has been found to cause cell transformation in transfected fibroblasts (Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., Dalla, Z. P., Giordano, S., Graziani, A., Panayotou, G., and Comoglio, P. M. (1994) Cell 77, 261-271). We inserted this sequence into a mutant platelet-derived growth factor receptor (F5) to determine if this region of c-met can initiate cell motility and which signaling pathways it activates. The platelet-derived growth factor (PDGF) receptor/c-met hybrid (F5 met) initiated PDGF-dependent chemotaxis in renal epithelial cells (8.0 ± 2.3 versus 70.5 ± 4.8 cells/mm²), while the parental construct, F5, did not. Addition of PDGF to cells expressing F5 met caused activation of the phosphatidylinositol (PI) 3-kinase (control 2.0 ± 0.8, +PDGF 17.1 ± 5.1, n = 3, p < 0.05) and phospholipase C (control 478.5 ± 67 dpm/well, +PDGF 1049.3 ± 93, n = 4, p < 0.003), while neither pathway was activated in cells expressing F5. The chemotactic response of F5 met was inhibited by both the PI 3-kinase inhibitor wortmannin and the phospholipase C inhibitor U-71322. Selective activation of the PI 3-kinase utilizing a PDGF receptor mutant (F3) containing the native high affinity PI 3-kinase binding site also resulted in PDGF stimulated chemotaxis, although less than that generated by the c-met sequence.

These findings demonstrate that the 11-amino acid sequence from c-met initiates epithelial motility via co-incident activation of the PI 3-kinase and phospholipase C and that selective activation of the PI 3-kinase can initiate a partial chemotactic response.

Hepatocyte growth factor (HGF) has been independently characterized by its ability both to induce mitogenesis (1) and to cause “scattering” of epithelial cells in culture (2). Further study has implicated HGF and its high affinity receptor, c-met, in diverse biologic events that involve the motile response: chemotaxis and branching tubulogenesis in renal epithelial cells (3, 4) and wound healing and angiogenesis in endothelial cells (3, 5). The motile response induced by c-met is a complex one, which involves dissociation of cell contacts, ruffling of the leading edge, extension of lamellipodia, and finally migration (6). The role of c-met in this series of events has not yet been well characterized, but it is likely to involve the integration of multiple signaling pathways.

Examination of the COOH-terminal region of c-met reveals a single 11-amino acid sequence, Y1348-VHVNTY1356-VNV, which is predicted to be a low affinity site for the two SH2 domains of phosphatidylinositol Cγ (PLCγ) and the phosphatidylinositol 3-kinase (PI 3-K), and a high affinity site for the ras activating complex GRB2-hsos1 (7). No other domain outside of the tyrosine kinase region of c-met is predicted to bind any known signaling proteins, making it possible that this 11-amino acid domain is a site for competitive interaction of many, if not all, of the receptor’s signaling proteins. Indeed, Ponzetto et al. (8) were able to demonstrate co-immunoprecipitation of overexpressed c-met with PLCγ, GRB2-hsos1, and PI 3-K as well as pp60src (8). Altering both tyrosines in this 11-amino acid sequence eliminated association with all four proteins, leading these researchers to term this region a “multifunctional docking site.” Thus, this 11-amino acid sequence might be sufficient to activate all of the signaling activity induced by HGF and mediate the motility response.

Work in our laboratory has demonstrated the importance of the PI 3-K in HGF-mediated motility (4). Using the PI 3-K inhibitors wortmannin and LY-294002, we found striking inhibition of both motogenesis and morphogenesis. These experiments demonstrate that c-met-mediated activation of the PI 3-K is required for the full chemotactic response, but they do not address whether the PI 3-K alone is sufficient to initiate chemotaxis.

In this report, we used an altered platelet-derived growth factor receptor (PDGFR), which is unable to bind to PI 3-K, RasGAP, Syp/PTP, or PLCγ (9) as a vehicle to introduce and study the role of the 11-amino acid c-met sequence in the motility response of epithelial cells. Use of a PDGFR mutant in our inner medullary collecting duct cells (which do not express endogenous PDGFR) makes it possible to study selective tyrosine phosphorylation of this met sequence in the setting of normal epithelial signaling machinery. In addition, a second PDGFR mutant with the native high affinity PI 3-K binding site (Y240MDM ... Y751VPM) intact but with Tyr → Phe substitutions of the tyrosines critical for association with RasGAP, Syp/PTP and PLCγ (F3) were utilized to examine more selective activation of the PI 3-K in epithelial chemotaxis.

MATERIALS AND METHODS

Cell Culture and Reagents—mIMCD-3 cells are a murine renal tubular epithelial cell line that expresses the c-met receptor and exhibits striking chemotaxis to a gradient of HGF (4, 10, 11). Screening of these cells by Northern analysis, polymerase chain reaction, and Western blots revealed no evidence of the PDGFR. In addition, mIMCD-3 cells did not demonstrate a mitogenic (data not shown) nor chemotactic response to PDGF (see Fig. 2). NIH 3T3 cells were used as a control for expression of the native PDGFR. All cells were cultured in Dulbecco’s modified Eagle’s medium with 5% fetal calf serum using standard...
techniques. PDGF (Upstate Biotechnology, Inc.) and HGF (a gift from Dr. George Vande Woude) were used in concentrations from 1 to 100 ng/ml (Invitrogen, CA) to induce chemotaxis detected at 10 ng/ml in NIH 3T3 cells for PDGF and 40 ng/ml in mIMCD-3 cells for HGF. In addition, recombinant HGF (Institute of Immunology Co., Ltd., Tokyo, Japan) was tested with equivalent results. Wortmannin was obtained from Sigma; U-71322 was from Boehringer Mannheim. Creation of PDGF Receptor Clones—Two PDGF receptor mutants (F5, in which the tyrosine residues at 740, 741, 751, 1009, and 1021 have been replaced by phenylalanines, and F3, in which the residues at 740 and 751 remain tyrosines) were provided to us as a gift by Dr. Andrius Kazlauskas (9). The kinase insert region of the F5 PDGFR was then modified to enclose novel Sall and Aat1 restriction sites and allow insertion of the 33-nucleotide cDNA sequence encoding the 11-amino acid c-met sequence YVHVNATYVNV from the c-met acid motif YVHVNATYVNV from the c-kinase binding site of the PDGFR and chemotaxis in response to a PDGF gradient. None of the F5 clones tested exhibited PDGF-dependent chemotaxis; while F5 met clones did chemotax toward PDGF.

In Vivo Phosphoinositide 3-Kinase Assay—Immunoprecipitation of the PI 3-kinase was performed as described previously (11). Briefly, following 24 h of serum deprivation, confluent cells were stimulated with ligand and lysed as above. Aliquots of supernatant containing equal amounts of protein were immunoprecipitated for 3 h at 4 °C with monoclonal antibody directed against phosphotyrosine using protein A-Sepharose beads (Sigma). Beads were washed and incubated for 10 min at room temperature in kinase buffer (62.5 mM ATP, 6.25 mM MgCl₂, 1.25 mM Hepes, 0.25 mg/ml phosphatidylinositol (Avanti Polar Lipids), 50 μCi [γ⁻³²P]ATP, pH 7.0). Lipids were then extracted and separated on oxalate-coated thin-layer chromatography plates (EM Science), and autoradiography was performed. The labeled lipids migrating as phosphatidylinositol 3-phosphate (PI3P) were then cut out and counted in a β scintillation counter for quantification of amount of PI3P generated.

In Vitro Phospholipase C Assay—24-well plates of either F5 or F5 met cells were incubated with inositol-free medium and 5 μCi/ml [³²P]inositol for 48 h. Cells were then washed twice in PBS, followed by a 20-min incubation with inositol-free media. PDGF or vehicle control was added to appropriate wells for 2 min. Cells were lysed with ice-cold trichloroacetic acid, and samples were extracted with water-saturated ether, dried in a vacuum concentrator, and separated by HPLC by the method of Auger et al. (13).

Statistical Analysis—Results were averaged, and statistical relevance was determined by the Student's t test. Data are presented as mean ± S.E.

RESULTS

Cells Expressing F5 met Chemotax in Response to PDGF—G418-resistant clones from transfections with the F5 and F5 met constructs were simultaneously screened for expression of the PDGFR and chemotaxis in response to a PDGF gradient. None of the F5 clones tested exhibited PDGF-dependent chemotaxis (Fig. 2), regardless of the level of expression of the full-length PDGF protein. One clone, F5e, which had the highest level of PDGF expression, was then utilized as a control for further experiments. Evaluation of the F5e clone revealed no chemotaxis in response to PDGF (control, 6.3 ± 4.0 cells/mm², n = 4; PDGF, 2.7 ± 1.2 cells/mm², n = 4; p = not significant), despite a full chemotactic response to HGF (154 ± 17.3, p < 0.001).

In contrast, we identified eight F5 met clones that demonstrated chemotaxis in response to PDGF and found that all eight clones expressed the PDGFR. Additionally, no clone exhibited PDGF-dependent chemotaxis, which did not express the receptor. Among these clones, the amount of chemotaxis correlated well with the level of expression of the PDGFR (Fig. 2).

![Fig. 1. Schematic depiction of the PDGF receptor mutants.](https://example.com/fig1)

![Fig. 2. Directional chemotaxis of mIMCD-3 cell clones toward a PDGF gradient.](https://example.com/fig2)
An 11-Amino Acid Sequence of c-met Mediates Chemotaxis

3). Interestingly, clones F5 met 29 and F5 met 41 subsequently failed to chemotax to PDGF and were found to no longer express the PDGF by Western analysis. One clone, F5 met 23, which stably expressed the mutant PDGFR, was used for further experiments and displayed consistent ability to chemotax toward PDGF (control, 4.6 ± 3.5 cells/mm², n = 9; PDGF, 78.6 ± 12.7 cells/mm², n = 11; p < 0.001).

F5 met Chemotaxis Correlates with Ligand-dependent Association with the PI 3-K—Following demonstration that the F5 16 and F5 met 23 clones exhibited similar levels of receptor protein expression and PDGF-dependent tyrosine phosphorylation (Fig. 4), we examined the ability of these clones to associate with the active form of the PI 3-K (Fig. 5). Antiphosphotyrosine immunoprecipitates of F5 met 23 cells revealed a marked increase in receptor association with the active form of the PI 3-K—

The importance of the association of the mutant PDGFR with the PI 3-K in the initiation of chemotaxis was confirmed by incubation of the cells with the PI 3-K inhibitor wortmannin. In these experiments, 10 nM wortmannin was found to markedly inhibit PDGFR-dependent chemotaxis in F5 met cells (+PDGF, 179.1 ± 42.1 versus +PDGF + 10 nM wortmannin, 70 ± 10.3, n = 9, p = 0.023).

Activation of PLC Contributes to the Chemotactic Response—In vitro study of inositol phosphate production in F5 met 23 cells revealed a doubling of inositol 1,4,5-trisphosphate production following PDGF stimulation (control 478.5 ± 67 dpm/well, +PDGF 1049.3 ± 93, n = 4, p = 0.003) (Fig. 6). There was no increase noted in F5 26 (control 860.8 ± 124 dpm/well, +PDGF 887.3 ± 104, n = 4, p = not significant). These results demonstrate that the 11-amino acid c-met sequence activates PLC in a ligand-dependent manner.

A PLC inhibitor, U-73122 was then tested for its effect on F5 met chemotaxis (14). U-73122 had no effect on the activity of the PI 3-K (control 126.9 ± 37 dpm PI3P/μg; 1 μM U-73122, 144.8 ± 46 dpm/μg, n = 3; p = not significant) and did not inhibit chemotaxis initiated by the protein kinase C activator diacylglycerol (diacylglycerol 142.5 ± 42.4 cells/mm², n = 12; diacylglycerol + U-73122, 131.7 ± 9.6, n = 9; p = not significant). However, at concentrations as low as 10 nM, there was a 70% inhibition of PDGF dependent chemotaxis, supporting a major role for PLC in c-met mediated chemotaxis (Fig. 7).

Selective Activation of the PI 3-K Initiates a Chemotactic Response—To examine the effect of selective activation of the PI 3-K on cell motility, we also transfected mLMC-3 cells with a mutant PDGFR receptor which has Tyr → Phe substitutions at binding sites for PLC, SyrPTP, and RasGAP but with the high affinity binding site for PI3-K intact (F3). Four G418-resistant F3 clones were identified that expressed the receptor and demonstrated PDGFR-dependent chemotaxis (Table I). Again, all clones that expressed the receptor at high levels were found to chemotax to PDGF, and no clones demonstrated chemotaxis in the absence of receptor expression. As expected, the

**FIG. 4.** Expression and phosphorylation of mutant PDGFR in mLMC-3 cell clones. a, whole cell lysates from confluent plates of cells were analyzed with an anti-PDGFR Western blot. The 190-kDa PDGFR receptor is evident in all but parent IMCD cells. b, mutant PDGFRs are tyrosine-phosphorylated in response to PDGF. Lysates of PDGF-stimulated or unstimulated mLMC-3 cells and PDGFR clones were immunoprecipitated with anti-PDGFR. Complexes were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine.

**FIG. 5.** PDGF stimulated PI 3-K activity in IMCD cells expressing the PDGFR mutants. Cells were stimulated with PDGF or vehicle control, immunoprecipitated with an antibody to phosphotyrosine, and then incubated with phosphatidylinositol (PI) substrate and [γ-32P]ATP. Labeled lipids were separated by thin-layer chromatography (top panel), and the PI3P was cut out and counted in a β scintillation counter and expressed in disintegrations/min/g of protein immunoprecipitated (bottom panel). Results are the mean of three separate experiments. *, p < 0.05 in comparison with unstimulated control; **, p < 0.025 in comparison with unstimulated control.
FIG. 6. HPLC analysis of inositol phosphate from F5 met cells. F5 met cells were labeled with [3H]inositol as described and stimulated for 2 min with either vehicle control or 10 ng/ml PDGF. Cytosolic lipids were then separated via HPLC. Inositol 1,4,5-phosphate eluted at 28.3 min.

FIG. 7. Inhibition of PLC blocks F5 met-mediated chemotaxis. Directional chemotaxis of IMCD cells toward a gradient of either 10 ng/ml PDGF or 40 ng/ml HGF with and without 10 μM U-73122, a PLC inhibitor.

**TABLE I**

| Cell type | Relative PDGF receptor expression | Chemotaxis | PDGF dependent PI 3-K activity |
|-----------|----------------------------------|------------|--------------------------------|
| 3T3       | ++++                             | 6.7 ± 2.3  | 156.0 ± 17.4 45.85 |
| IMCD-3    | –                                | 0.1 ± 0.1  | 0.4 ± 0.5   0.02 |
| F3-1      | ++++                             | 4.2 ± 1.1  | 50.0 ± 4.5  28.49 |
| F3-9      | +                                | 2.1 ± 1.3  | 11.8 ± 6.6  1.30 |
| F3-17     | –                                | 2.8 ± 0.2  | 0.1 ± 0.1   0.00 |
| F3-20     | ++                               | 7.0 ± 2.3  | 18.9 ± 4.0  10.54 |
| F3-31     | +++                              | 2.9 ± 0.8  | 18.7 ± 3.4  24.40 |

*By western blot in comparison to 3T3 cells.

**DISCUSSION**

The potent chemotactic effect of HGF on epithelial cells makes the study of its receptor, c-met, a particularly relevant system for determining the signaling cascade involved in the epithelial motility response. The use of a hybrid F5-c-met receptor (F5 met) allowed us to selectively examine the role of an 11-amino acid region of c-met in epithelial cell motility and signal transduction.

We initially determined that all three of the transfected receptor constructs were successfully expressed in mIMCD-3 cells and underwent PDGF-dependent tyrosine phosphorylation. The F5 mutant PDGFR, which does not bind PI 3-K, PLCγ, PTP, or RasGAP but is still capable of associating with src (via Y579FV) and possibly GRB2 (via Y716FNA) (15) did not initiate epithelial chemotaxis in response to PDGF. However, expression of the F5 met hybrid receptor did initiate chemotaxis in a ligand-dependent manner, demonstrating that phosphorylation of the inserted c-met sequence Y349FVHNATYVNV activated those signaling pathways necessary for epithelial cell motility.

Based on our observation that the PI 3-K inhibitors wortmannin and LY294002 could partially block HGF/c-met-mediated epithelial chemotaxis (4), as well as data from Kundra and co-workers (16) that demonstrated that PDGFR constructs excluding PLC activation exhibited diminished chemotactic responses (16), we examined the ability of the 11-amino acid c-met sequence to activate these two candidate signaling pathways in epithelial cells. Indeed, both the PI 3-K and PLC were activated in a ligand-dependent manner by the F5 met hybrid receptor, but not by the parental F5 construct. The importance of these two pathways in chemotaxis was then examined using inhibitors of PI 3-K and PLC as well as a receptor construct, which selectively activates PI 3-K. Both wortmannin, an inhibitor of PI 3-K, and U-73122, an inhibitor of PLC, caused marked reductions of F5 met-stimulated chemotaxis. Thus, the PI 3-K and PLC are activated in vivo by the 11-amino acid sequence from c-met and contribute to the chemotactic response initiated by this receptor.

In addition, the F3 PDGFR in which the native high affinity PI 3-K binding site (Y740 MDM...Y751VPM) has been selectively restored (but which still lacks PLC, RasGAP, and PTP binding sites) also caused epithelial chemotaxis in response to PDGF. It should be noted that one study has described the small adaptor molecule Nck as competing for binding to the Y751FV sequence of F3 as well (17). The above data demonstrate a critical role for both the PI 3-K and PLC in epithelial cell migration and are the first to show that selective activation of PI 3-K is sufficient to initiate chemotaxis.

Our findings are complemented by data from several laboratories. Wennstrom et al. (18) examined the ruffling response in porcine aortic endothelial cells transfected with a mutant PDGFR, which selectively excluded binding of the PI 3-K (Tyr → Phe substitutions at 740 and 751) (8). This construct, the opposite of our F3 PDGFR, failed to mediate ruffling in response to PDGF, implicating a requirement for PI 3-K in membrane ruffling. Likewise, Kundra et al. (16) examined the same Y740F/ Y751F mutation in chemotactic assays of a canine kidney epithelial cell line (16). Again, the chemotactic response was obliterated by an inability to activate the PI 3-K, as well as...
a mutant excluding activation of PLC. Here, we demonstrate that selective activation of PI 3-K initiates a chemotactic response, while coactivation of PI 3-K and PLC produces a marked increase in this response.

Other signaling pathways important in cell movement involve the small GTP binding proteins Ras, Rac and Rho. In a recent study by Ridley et al. (19), microinjection of Madin-Darby canine kidney cells with constitutively active Ras reproduced HGF-mediated ruffling but did not initiate cell scattering (19). Thus, it may be that isolated activation of ras is sufficient to generate actin filament rearrangements but not actual cell movement. In agreement with this, our F5 PDGFR clones did not mediate PDGF-dependent chemotaxis, even though ras activation may occur via GRB2/hSos1 nucleotide exchange factor association at Y716SNA in the F5 receptor (20). However, since both the F5 met and F3 receptors are capable of Grb2/hSos1/Ras activation, we cannot rule out a contributory or co-stimulatory effect of Ras activation on chemotaxis in these experiments.

The c-met receptor mediates diverse phenotypic changes in several cell types including cell motility and tubule formation. An 11-amino acid sequence from this receptor, when phosphorylated in a ligand dependent manner, activates both PLC and the PI 3-K, triggering the intracellular events necessary for epithelial chemotaxis.

Acknowledgments—We thank Andrius Kazlauskas for the generous gift of mutant F5 and F3 PDGFR cDNAs as well as the antibody to the PDGFR. LY-294002 was generously provided by Chris Vlahos. We thank Donna Faletto and George Vande Woude for providing the HGF and anti-c-met antibody, and Steve Gullans for providing the IMCD-3 cells.

REFERENCES

1. Igawa, T., Kanda, S., Kanetake, H., Saitoh, Y., Ichihara, A., Tomita, Y., and Nakamura, T. (1991) Biochem. Biophys. Res. Commun. 174, 831–838
2. Stoker, M., Gherardi, E., Perryman, M., and Gray, J. (1987) Nature 327, 239–242
3. Montesano, R., Schaller, G., and Ordi, L. (1991) Cell 66, 697–711
4. Derman, M., Cunha, M., Barros, E., Nigam, S., and Cantley, L. (1995) Am. J. Physiol. 269, F1211–F1217
5. Bussolino, F., Di, R. M., Ziche, M., Bocchiotto, E., Olivero, M., Naldini, L., Gaudino, G., Tamagnone, L., Coffer, A., and Comoglio, P. M. (1992) J. Cell Biol. 132, 629–641
6. Ridley, A. J. (1994) Bioessays 16, 321–327
7. Cantley, L., and Cantley, L. (1995) J. Am. Soc. Nephrol. 5, 1872–1881
8. Porzotto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G., and Comoglio, P. M. (1994) Cell 77, 261–271
9. Valius, M., and Kazlauskas, A. (1993) Cell 73, 321–334
10. Rauchman, M., Nigam, S., Delpire, E., and Gullans, S. (1993) Am. J. Physiol. 34, F416–F424
11. Cantley, L., Barros, E. J. G., Gandhi, M., Rauchman, M., and Nigam, S. K. (1994) Am. J. Physiol. 267, F271–F280
12. Stoker, M. (1989) J. Cell. Physiol. 139, 565–569
13. Auger, K. R., Serunian, L. A., Sottoff, S. P., Libby, P., and Cantley, L. C. (1989) Cell 57, 167–175
14. Bleasdale, J. E., and Fisher, S. K. (1993) Neuroprotocols 3, 125–133
15. Claesson-Welsh, L. (1994) J. Biol. Chem. 269, 32023–32026
16. Kundra, V., Escobedo, J., Kazlauskas, A., Kim, H., Rhee, S., Williams, L., and Zetter, B. (1994) Nature 367, 464–476
17. Nishimura, R., Li, W., Kashishian, A., Mondino, A., Zhou, M., Cooper, J., and Schlessinger, J. (1993) Mol. Cell. Biol. 13, 6889–6896
18. Wennstrom, S., Siegbahn, A., Yokote, K., Arvidsson, A. K., Heldin, C. H., Mori, S., and Claesson, W. L. (1994) Oncogene 9, 651–660
19. Ridley, A., Comoglio, P., and Hall, A. (1995) Mol. Cell. Biol. 15, 1110–1122
20. Arvidsson, A., Rupp, E., Nanberg, E., Downward, J., Ronnstrand, L., Wennstrom, S., Schlessinger, J., Heldin, C., and Claesson-Welsh, L. (1994) Mol. Cell. Biol. 14, 6715–6726
An 11-Amino Acid Sequence from c-met Initiates Epithelial Chemotaxis via Phosphatidylinositol 3-Kinase and Phospholipase C
Melanie P. Derman, Jean Y. Chen, Katherine C. Spokes, Zhou Songyang and Lloyd G. Cantley

J. Biol. Chem. 1996, 271:4251-4255.
doi: 10.1074/jbc.271.8.4251

Access the most updated version of this article at http://www.jbc.org/content/271/8/4251

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 20 references, 6 of which can be accessed free at http://www.jbc.org/content/271/8/4251.full.html#ref-list-1