Hepatocyte growth factor (HGF), also known as scatter factor, is a member of the plasminogen-related growth factor family and is a key mediator of cell migration, proliferation, survival, motility, and morphogenesis (1–4). HGF is known to specifically activate the Met receptor tyrosine kinase, resulting in downstream activation of RAS and PI3K signaling pathways (5–7). Although HGF/Met signaling is critical for processes such as wound healing and tissue regeneration (8–10), overstimulation of this pathway can drive tumor growth in multiple cancers (3, 11, 12). As a result, understanding of the molecular mechanism for HGF activation of the Met receptor has received considerable attention with the aim of devising novel strategies for either enhancing or inhibiting HGF-dependent Met signaling, depending on the therapeutic context (13–18).

HGF is a secreted extracellular protein that exists as an inactive single-chain ligand (pro-HGF) until proteolytic cleavage at the Arg^494-Val^495 peptide bond results in a two-chain form, consisting of a disulfide-linked α/β-heterodimer, capable of activating the Met receptor (1, 19–22). The domain architecture of HGF is analogous to that of plasminogen, where the α-chain comprises an N-terminal domain (N) followed by four Kringle domain repeats (K1–K4) and the β-chain contains the C-terminal trypsin-like serine protease domain (Fig. 1A) (23, 24). Interestingly, both two-chain HGF and pro-HGF are capable of high affinity binding to the Met receptor through specific interactions with the α-chain; however, receptor activation can only occur after cleavage of pro-HGF into the two-chain form (19, 25, 26).

Extensive structural and biochemical work on trypsin-like serine proteases has defined a conserved activation mechanism involving cleavage of the zymogen and subsequent rearrangement of three loops and the newly formed N terminus, collectively termed the “activation domain” (27, 28). In this mechanism, the new N terminus inserts into a canonical “activation pocket,” resulting in conformational changes in the loops and allosteric regulation of the active site for catalysis. Structural and biochemical studies have revealed that HGF utilizes this activation mechanism for Met signaling (29–31). In HGF, the newly formed N terminus (Val^495) in the β-chain, which corresponds to residue 16 in chymotrypsin numbering, inserts into a canonical “activation pocket” (Fig. 1B). N-terminal insertion is stabilized by hydrophobic interactions with the Val^495 side chain and electrostatic interactions between the positively charged N terminus and the negatively charged Asp^672 residue (Asp^194 in chymotrypsin numbering), which is highly conserved in trypsin/chymotrypsin-like serine proteases. Previous work has shown that mutation of the N-terminal Val^495 to Gly or Asp^672 to Asn within the β-chain prevents N-terminal insertion, thereby disrupting β-chain interactions at the Arg^494-Val^495 peptide bond in the zymogen-like pro-HGF results in allosteric activation of the serine protease-like β-chain (HGF β), which binds Met to initiate signaling. We use insights from the canonical trypsin-like serine protease activation mechanism to show that isolated peptides corresponding to the first 7–10 residues of the cleaved N terminus of the β-chain stimulate Met phosphorylation by pro-HGF to levels that are ~25% of those stimulated by two-chain HGF. Biolayer interferometry data demonstrate that peptide VVNGIPTR (peptide V8) allosterically enhances pro-HGF β binding to Met, resulting in a KD of 1.6 μM, only 8-fold weaker than the Met/HGF β-chain affinity. Most notably, in vitro cell stimulation with peptide V8 in the presence of pro-HGF leads to Akt phosphorylation, enhances cell survival, and facilitates cell migration between 75 and 100% of that found with two-chain HGF, thus revealing a novel approach for activation of Met signaling that bypasses proteolytic processing of pro-HGF. Peptide V8 is unable to enhance Met binding or signaling with HGF proteins having a mutated activation pocket (D672N). Furthermore, Gly substitution of the N-terminal Val residue in peptide V8 results in loss of all activation.

Overall, these findings identify the activation pocket of the serine protease-like β-chain as a “hot spot” for allosteric regulation of pro-HGF and have broad implications for developing selective allosteric activators of serine proteases and pseudoproteases.
binding to Met and completely abolishing the signaling activity of two-chain HGF (31). Thus, the key mechanistic step for conversion of pro-HGF into a Met agonist is directly analogous to the activation of trypsin-like serine proteases (28), despite the fact that the serine protease-like β-chain mediates protein-protein interactions rather than proteolytic activity.

In a seminal paper in 1976, dipeptides corresponding to the first 7–10 residues of the HGF β-chain N terminus can activate single-chain HGF (scHGF), a noncleavable mutant of pro-HGF (22), to stimulate Met phosphorylation. This step was omitted for scHGF, which contained the R424A and R494E mutations to prevent proteolytic processing (22). All mutations were introduced by quantitative amino acid analysis.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Full-length HGF Proteins**—Full-length recombinant HGF proteins were expressed in 1-liter fermentation cultures of Chinese hamster ovary (CHO) cells and purified as described previously (22). Briefly, cells were grown for 13 days in expression medium (F-12/Dulbecco’s modified Eagle’s medium supplemented with 1% ultralow IgG fetal bovine serum [FBS]). Expression medium containing full-length HGF proteins was incubated an additional 2–3 days with 5–10% FBS at 37 °C to allow for proteolytic conversion to two-chain HGF. This step was omitted for scHGF, which contained the R424A and R494E mutations to prevent proteolytic processing (22). All mutations were introduced using QuikChange™ site-directed mutagenesis (Stratagene) and verified by DNA sequencing. Proteins were purified using HiTrap-Sepharose SP cation exchange chromatography as described (22). SDS-PAGE (4–20% gradient gel) analysis under reducing conditions revealed that the proteins were >95% pure, and only the two-chain HGF forms could be resolved into α/β heterodimers. Protein concentration was determined by quantitative amino acid analysis.

**HGF β Proteins and Peptides**—The HGF β-chain construct used (residues Val495–Ser728) contains the C604S mutation, and the scHGF β-chain (residues Asn479–Ser728) contains the R494E mutation, both described previously (30). The scHGF β-chain has Cys604 to allow for disulfide formation between Cys487 and Cys604. All mutations were introduced using QuikChange™ site-directed mutagenesis (Stratagene) and verified by DNA sequencing. Proteins were expressed as C-terminal His tag fusions from the pAcGP67 vector (BD Biosciences) in insect cells and purified as described previously (22). Briefly, Sf9 cells were plated and transfected in ESF 921 medium (Expression Systems LLC, Woodland, CA) using the BaculoGold™ expression system according to the manufacturer’s instructions (Pharmingen). Virus was generated through three rounds of amplification, and 10 ml of the round 3 stock was used to infect 1 liter of High Five™ insect cells (Invitrogen) at a density of 1 × 10⁶ cells/ml. Cells were grown through three rounds of amplification, and 10 ml of the round 3 stock was used to infect 1 liter of High Five™ insect cells (Invitrogen) at a density of 1 × 10⁶ cells/ml. Cells were grown
for 72 h at 27 °C and removed from the medium by centrifugation at 3000 × g for 15 min. Supernatant was removed from cells and supplemented with 1 mM NiCl₂, 5 mM CaCl₂, in 50 mM Tris-HCl, pH 7.5, final concentrations. Supernatant was then filtered through 0.2-µm filter, applied to a 3-ml Ni²⁺-NTA column (Qiagen) using gravity flow, washed with 30 ml of wash buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 mM imidazole), and eluted off the column with elution buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 300 mM imidazole). Protein was concentrated to 1 ml and loaded onto a SuperdexS75 gel filtration column (GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl. Fractions containing HGF β proteins were analyzed by SDS-PAGE and pooled, and concentration was determined by the absorbance at 280 nm. N-terminal sequence analysis revealed correct N termini following the His8 tag, using the corresponding restriction sites. An AviTag DNA sequence was cloned into the pFastBac1 vector (Invitrogen) using NotI, removed from the pAcGP67 vector (BD Biosciences), and subcloned into the corresponding restriction sites. An AviTag DNA sequence (corresponding to amino acids GLNDIFEAQKIEWHE) was used in the Bac-to-Bac system (Invitrogen) to generate recombinant baculovirus particles. Peptide Activators of Pro-HGF Stimulate Met Signaling

Met Phosphorylation Kinase Receptor Activation Assay (KIRA)—The KIRA was carried out using lung carcinoma A549 cells (CCL-185, ATCC, Manassas, VA) essentially as described previously (29). In all cases, Met phosphorylation activity was determined following a 10-min stimulation at 37 °C with 100 ng/ml HGF, scHGF, or mutant protein in medium containing 0.1% FBS. For peptide activation experiments, 2 mM peptide or a dose titration of peptide V8 was incubated with 100 ng/ml protein for 30 min prior to the addition to cells. Met phosphorylation is reported as a percentage of maximal signal obtained with two-chain HGF.

Measurement of HGF β Binding to Met ECD Using Biolayer Interferometry—Met binding assays were carried out at 30 °C in 1× kinetics buffer (ForteBio, Inc.) containing PBS and utilized the OctetRed™ biosensor instrument (ForteBio, Inc.). In all cases, 25 µg/ml biotinylated Met ECD (Sema/PSI-AviTag) was captured on the surface of streptavidin optical sensor tips (SA biosensors, ForteBio, Inc.), washed, and transferred into buffer containing HGF β-chain variants in the presence or absence of the indicated peptides. For binding reactions containing peptides, a 30-min preincubation of protein and peptide was performed at room temperature before binding was measured. Association reactions were monitored for 15 min until the binding reached steady state. Subsequently, the bound material was transferred into reaction buffer, and dissociation reactions were monitored for 15 min to ensure reversible binding between HGF β proteins and Met. Binding was quantified using the steady-state levels of surface response from the association reaction. The effects of peptides on HGF β-chain variant binding to Met were measured using 0.5 µM protein in the presence of a 5 mM concentration of the indicated peptide or increasing concentrations of peptide V8 to determine the apparent binding affinity. Equilibrium constants were derived by least squares fitting of the titration data to a single-site binding equation. Errors reported are ±S.D. of n = 3 independent experiments.

Western Blot Analysis—PC3 human prostate cancer cells were obtained from ATCC (Manassas, VA) and maintained in 50:50 Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (Invitrogen) supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. For phosphorylation studies, subconfluent cells were serum-starved for 1 h in medium containing 0.5% BSA. After 1 h, cells were stimulated with 100 ng/ml two-chain HGF, 200 ng/ml scHGF, or 200 ng/ml HGF mutant in the presence or absence of peptide for 10 min at 37 °C. Control wells contained either 0.04% DMSO or peptide alone. Samples were prepared by washing the cells twice with ice-cold PBS followed by the addition of SDS-PAGE sample buffer. Samples containing 25 µg of protein were electrophoresed under reducing conditions on 4–12% Tris-glycine gels (Invitrogen), transferred to nitrocellulose membranes, and blocked with Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE) for 1 h. To determine Met and Akt phosphorylation, the membrane was probed with Met monoclonal antibody, extracellular region, clone DL-21 (Upstate Biotechnology, Inc., Lake Placid, NY), phospho-Met polyclonal antibody (Tyr1234/1235) (Cell Signaling Technology, Danvers, MA), Akt polyclonal antibody (Cell Signaling Technology), and phospho-Akt monoclonal antibody (Ser473) (587F11) (Cell Signaling Technology) overnight at 4 °C. After washing, the membrane was probed with alkaline phosphatase-coupled secondary antibody, and the membrane was developed with BCIP/NBT substrate solution.
incubated with IRDye™800-conjugated goat anti-mouse IgG (Rockland, Gilbertsville, PA) and AlexaFluor® 680 goat anti-rabbit IgG (Invitrogen) for 1 h. The amounts of phosphorylated and total kinase expression were detected using the Odyssey® infrared imaging system (Li-Cor Biosciences). The data have been background-subtracted and are represented as a ratio of phosphorylated to non-phosphorylated protein.

Cell Survival Assay—Cal-51 human breast cancer cells were obtained from ATCC and maintained in cell culture medium (RPMI, 10% FBS (Sigma), 50 units/ml penicillin, 50 µg/ml streptomycin, 2 mM glutamine) and used between passages 2 and 12. For the survival assay, cells were plated at 10,000 cells/well in the 60 inner wells of Microtest 96-well tissue culture plates (Falcon 353072) in 50 µl of assay diluent (RPMI, 0.1% BSA (Sigma) 50 units/ml penicillin, 50 µg/ml streptomycin, 2 mM glutamine). Cells were allowed to attach for 1–2 h in a 37 °C CO2 incubator. Final concentrations of HGF variants and peptides were 500 ng/ml and 1 µM, respectively. The DMSO concentration used for peptide experiments had no effect on cell viability. After 72 h, 25 µl of alamarBlue® (BUD012B, Serotec) was added to each well and incubated for an additional 5–7 h at 37 °C in a CO2 incubator to allow fluorescent product to develop. Plates were agitated vigorously for 10 min at room temperature, and fluorescence was read at 530–590 nm in a fluorescent plate reader.

Scratch Assay—HeLa human epithelial carcinoma cells were from ATCC and maintained in culture medium (DMEM, 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine) at 37 °C in a CO2 incubator. Cells between passages 4 and 9 were grown to ~80% confluence in T175 flasks, lifted in PBS containing 0.05% trypsin (Invitrogen), and plated at 175,000 cells/well in 24-well ImageLock plates. Cells were allowed to adhere overnight at 37 °C and formed confluent monolayers. Scratches within the cell monolayers (~600 µm wide) were precisely generated through the center of the wells using the WoundMaker apparatus (Essen Biosciences, Inc.), washed twice in PBS to remove cell debris, and replaced with culture medium containing 0.2% FBS as a negative control or medium containing 0.2% FBS plus either 100 ng/ml two-chain HGF, 100 ng/ml scHGF, 100 ng/ml scHGF plus 2 mM peptide V8, or 2 mM peptide V8. Repopulation of the denuded monolayer by migrating cells was then imaged for at least 30 h at a rate of 1 frame/h in the IncuCyte imaging instrument (Essen Biosciences, Inc.) using time-lapse bright field microscopy. Data analysis was carried out with the IncuCyte software to quantify the total accessible surface area within the scratch for each frame. The reduction in surface area was reported as increase in percentage of scratch closure. Error in the data is reported as ±S.D. of n = 4.

RESULTS

Peptides with the N-terminal Sequence of the HGF β-Chain Stimulate scHGF-dependent Phosphorylation of Met—To address whether peptides with the N-terminal sequence of the HGF β-chain (495VVNGIPTRTN504 . . . ) can activate scHGF in trans and signal through the Met receptor, a selected number of peptides of increasing lengths, starting from the dipeptide sequence VV (V2) and continuing up to the first 10 residues (V10), were synthesized (Table 1) and tested with various HGF mutants (Table 2) in a cell-based Met KIRA with A549 lung carcinoma cells. In this assay, we first tested the HGF R424A/R494E mutant (scHGF) as a zymogen-like non-cleavable form of pro-HGF that is specifically prevented from proteolytic conversion to the active two-chain HGF ligand (22). Neither 100 ng/ml scHGF by itself nor a 2 mM concentration of any of the N-terminal peptides alone were sufficient to stimulate Met phosphorylation (Fig. 2A). Peptides V2, V3, V4, and V6 individually added to scHGF also had no ability to activate scHGF-dependent Met phosphorylation. Given that peptide V6 is inactive, it is highly unlikely that peptide V5, which was unfortunately insoluble, would have Met phosphorylation activity. However, longer peptides (V7–V10) specifically enhanced scHGF-dependent Met phosphorylation

### TABLE 1
Sequences of HGF β-chain N-terminal peptides

| Peptide name | Sequence | HGF residue numbers |
|--------------|----------|---------------------|
| V2           | VV       | 495–496             |
| V3           | VVN      | 495–497             |
| V4           | VVNG     | 495–498             |
| V6           | VVNGIPT  | 495–500             |
| V7           | VVNGIPT  | 495–501             |
| V8           | VVNGIPTV | 495–502             |
| V9           | VVNGIPTV | 495–503             |
| V10          | VVNGIPTVRN| 495–504             |
| G8           | GVNGIPT  | 495–502             |
| V8A1         | AVNGIPT  | 495–502             |
| V8A2         | AVNGIPT  | 495–502             |
| V8A3         | AVNGIPT  | 495–502             |

### TABLE 2
Full-length and β-chain variants of HGF and scHGF that modulate insertion of the N terminus of the β-chain into its activation pocket

| Protein            | HGF residue numbers | N terminus of the HGF β-chain                                      |
|--------------------|---------------------|-------------------------------------------------------------------|
| HGF                | 1–728               | Inserted                                                          |
| HGF (V495G)        | 1–728               | Exposed (N-terminal β-chain mutant)                               |
| HGF (D672N)        | 1–728               | Exposed (activation pocket mutant)                                |
| HGF β              | 495–728             | Inserted (β-chain only)                                           |
| HGF β (V495G)      | 495–728             | Exposed (N-terminal mutant, β-chain only)                         |
| scHGF              | 1–728               | Exposed; non-cleavable (R494E and R424A mutations)                |
| scHGF β            | 479–728             | Exposed; non-cleavable (scHGF β with activation pocket mutant)    |
| scHGF β (D672N)    | 479–728             |                                                                  |

* Residues 495 and 672 refer to residues 16 and 194 in chymotrypsin numbering.

** HGF β and HGF β (V495G) also contain the C604S mutation to prevent any cysteine-mediated oxidation; Cys464 forms a putative disulfide with Cys467 in the α-chain.

---

**Peptide Activators of Pro-HGF Stimulate Met Signaling**

—HeLa human epithelial carcinoma cells were precisely enhanced scHGF-dependent Met phosphorylation.
These peptides stimulated phosphorylation to levels that were ~25% of the level measured using fully activated two-chain HGF. Thus, N-terminal peptides can stimulate scHGF-dependent phosphorylation of Met but require a minimum of 7 residues. Subsequent studies focused primarily on peptide V8 (VNVGIIPTR).

In addition to revealing the dependence on peptide length, Fig. 2A also suggests the mechanism of action for these peptides. In two-chain HGF, V495G or D672N mutations reduce Met signaling due to impaired N-terminal insertion into their activation pockets (31). Peptide V8 partially activated the HGF V495G mutant as shown by enhanced Met phosphorylation (Fig. 2A); however, it had no effect upon the HGF D672N mutant (Fig. 2A), suggesting that the peptide activation mechanism requires an exposed and functional activation pocket in the HGF β-chain. Furthermore, replacement of the N-terminal Val with Gly (peptide G8) completely abolished the activity of peptide V8, indicating that the hydrophobic side chain of the N terminus is also critical for activation of scHGF.

Titration of peptide V8 yielded a half-maximal stimulation of Met phosphorylation by both scHGF and HGF V495G of ~0.5 mM, whereas the D672N mutant was inactive at all concentrations of peptide V8 (Fig. 2B). The apparent inhibition seen at higher peptide concentrations has previously been observed with higher HGF concentrations as well (29). Taken together, these findings suggest peptides that minimally contain the first seven residues of the HGF β N-terminus can specifically activate scHGF in trans and implicate the activation pocket of the serine protease-like β-chain as the target for these peptides.

**N-terminal Peptides Increase the Binding Affinity of scHGF β to Met**—Because high affinity binding is governed by the α-chain binding to Met (26), quantifying the effect of N-terminal peptides on scHGF-dependent Met binding is difficult to interpret using full-length HGF. Therefore, we assessed the peptide activation mechanism using isolated ECD domains (Fig. 1A and Table 2). The finding that Asp<sup>672</sup> of the β-chain is required for N-terminal peptide activation of scHGF (Fig. 2, A and B) suggests that the peptides directly interact with the activation pocket of the β-chain and stabilize an “active-like” conformation that productively binds Met. To test this hypothesis, we measured the ability of a subset of the N-terminal peptides to enhance binding of a “zymogen-like” form of the isolated HGF β-chain (scHGF β) to the Met Sema/PSI domain. In this assay, biolayer interferometry was used to detect scHGF β binding to a surface-immobilized form of the Met receptor ECD (biotinylated Met Sema/PSI-AviTag).

As seen in Fig. 3A, of the three peptides tested (V4, V8, and G8), only peptide V8 enhanced binding of either scHGF β or HGF β V495G to Met by 7- and 5-fold, respectively, relative to scHGF β alone (Fig. 3A). No enhancement was observed for peptides V4 or G8 with any form of scHGF β. Furthermore, peptide V8 did not alter scHGF β D672N binding to Met, suggesting that the activation pocket within the scHGF β-chain is required. A limited alanine scan of peptide V8 (peptides V8A1, V8A2, and V8A3 in Table 1) resulted in dramatic reductions in the ability of peptide V8 to enhance scHGF β binding to Met (Fig. 3B). Alanine substitution at either of the first two valine residues resulted in a total loss of activity, whereas substitution of Asn at the third residue resulted in ~60% reduction in activity.

We then characterized peptide V8-dependent binding of scHGF β to the Sema/PSI domain of Met to determine the apparent <i>K<sub>D</sub></i> (K<sub>Dpp</sub>) value. A titration of 0.5 μM scHGF β with increasing concentrations of peptide V8 resulted in a K<sub>Dpp</sub> of 2 ± 1 mM (Fig. 3C), in reasonable agreement with the cell-based EC<sub>50</sub> of ~0.5 mM for V8 activation of full-length scHGF (Fig. 2B). Attempts to measure peptide V8 binding directly to scHGF β using biolayer interferometry as well as isothermal calorimetry were unsuccessful. This is not surprising given that the affinity is in the low millimolar concentration range. To further quantify the effect of peptide V8 on the scHGF β binding affinity for Met, we determined K<sub>Dpp</sub> values of 11 ± 1 and 1.6 ± 0.2 μM in the absence and presence of a near saturating amount of peptide V8 (5 mM), respectively (Fig. 3D). The peptide V8-bound form of scHGF β has an affinity for Met that is significantly shifted toward that of the HGF β-chain, which has a K<sub>Dpp</sub> of 0.20 ± 0.01 μM for Met binding (Fig. 3D, inset). Taken together, these results suggest that the N-termi-
minal peptide activators have specific hydrophobic and electrostatic interactions with the activation pocket of the scHGF β-chain and stabilize an active-like conformation with enhanced productive binding for Met.

Peptide-activated scHGF Promotes Cellular Signaling, Survival, and Migration—Previous work has shown that inactive scHGF variants are still capable of high affinity Met receptor binding (25, 26, 40), but these interactions do not elicit Met receptor phosphorylation or activation of downstream signaling because the β-chain does not effectively engage Met (31). Our observations suggest that the N-terminal peptides shift scHGF into a “pseudoagonist” state by allosterically activating β-chain for productive binding to Met. Given that peptide-activated scHGF results in partial stimulation of Met phosphorylation compared with HGF activity (Fig. 2, A and B), we wanted to confirm that the peptide-bound form of scHGF is a genuine Met agonist. To address this, we further analyzed receptor activation, Akt phosphorylation, and phenotypic cellular responses downstream of Met activation in multiple cell lines.

Quantitative Western blots show that only peptide V8 can “activate” scHGF or HGF V495G, which leads to phosphorylation of both Met and the downstream Akt effector kinase in PC3 prostate cancer cells (Fig. 4). In agreement with previous data, the HGF D672N mutant cannot be activated by peptide V8. The addition of peptide V8 to either scHGF or the HGF V495G mutant specifically stimulated Met phosphorylation in PC3 cells up to ~20% of the level of two-chain HGF, after accounting for background. Peptide V8-bound scHGF also stimulated Akt phosphorylation downstream of Met receptor activation in PC3 cells to levels comparable with those observed for two-chain HGF (Fig. 4, A and C). This may be a result of signal amplification of the phospho-Akt levels downstream of Met receptor phosphorylation and suggests that peptide V8-bound scHGF is capable of producing a phenotypic cellular response characteristic of Met signaling. Peptide V8-dependent phosphorylation of Met or Akt in the presence of scHGF or HGF V495G is also specific because peptides V4 or G8 are inactive (Figs. 4, B and C).

HGF-dependent Met signaling is a well known driver of cell survival and migration in many in vitro cellular systems (3, 41). Thus, we asked whether the peptide V8-bound form of scHGF could promote these cellular effects using Cal-51 breast cancer cells or HeLa cervical cancer cells. In cell survival assays, Cal-51 cells were incubated for a three-day period in the presence of scHGF, HGF V495G, or HGF D672N in combination with peptides V8 or G8 (Fig. 5A). Peptide V8 promoted cell survival in the presence of scHGF and HGF V495G that reached ~75 and 50% of the response compared
with two-chain HGF, respectively. Peptide V8 did not enhance cell survival for the HGF D672N activation pocket mutant, and peptide G8 had no effect for any of the HGF proteins. Finally, in scratch assays with HeLa cells, the addition of peptide V8 to scHGF resulted in enhanced cell migration that was comparable with the fully active two-chain HGF agonist (Fig. 5B). These findings are consistent with our biochemical data and demonstrate that peptide V8 is an agonist that can activate the pro-form of HGF in the absence of normal proteolytic cleavage, resulting in cellular responses downstream of Met signaling that are similar to those observed using two-chain HGF itself.

**DISCUSSION**

Non-proteolytic Allosteric Activation of Pro-HGF by N-terminal β-Chain Peptides—This study demonstrates a novel approach for activating the pro-form of HGF that bypasses the requirement for proteolytic processing to the active two-chain form. By utilizing peptides that mimic the role of the N terminus of the proteolytically activated HGF β-chain, we have significantly enhanced the activity of pro-HGF in Met and Akt phosphorylation, increased peptide-dependent binding of the scHGF β-chain to Met, and, most importantly, recapitulated cellular signaling in survival and migration that is comparable with two-chain HGF. Furthermore, we have shown that these processes require a functional activation pocket within the β-chain of pro-HGF that acts as a “hot spot” for allosteric regulation.

N-terminal insertion of the HGF β-chain into the canonical serine protease activation pocket strictly regulates productive β-chain binding to the Met receptor, required for HGF signaling (31). We hypothesized that a small peptide could recapitulate this mechanism when added in trans and elicit signaling by the “zymogen-like” form of pro-HGF, which tightly binds Met but cannot signal. Indeed, peptides corresponding to the first 7–10 residues of the activated β-chain N terminus are sufficient to elicit enhanced Met phosphorylation by scHGF. Using the binding of scHGF β to the Sema/PSI domain of Met as a readout for β-chain activation, we found that peptide V8 specifically increases its Met binding affinity, resulting in a $K_{D}^{app}$ of 1.6 µM, which is only 8-fold weaker than the Met/HGF β-chain affinity. The peptide binding data are in accord with
the Met phosphorylation results and implicate the β-chain as the target for peptide V8.

Activation by N-terminal Peptides Requires a Functional Activation Pocket—We have previously shown that the D672N and V495G mutations within the β-chain of two-chain HGF disrupt favorable electrostatic and hydrophobic interactions important for N-terminal insertion, leading to decreased insertion of the HGF β-chain N terminus and loss of HGF signaling activity (31). In accordance with this, peptide-dependent Met phosphorylation is completely abolished for the two-chain HGF D672N mutant or when using scHGF and peptide G8, where the N-terminal Val is changed to Gly (Fig. 2). Furthermore, peptide-dependent binding of the scHGF β-chain to Met is also abolished for the scHGF β-chain D672N mutant or when using peptide G8 (Fig. 3). Taken together, this provides strong evidence for a mechanism where the peptides act via insertion into the activation pocket of the HGF β-chain. The ability of these peptides to restore the activity of the HGF V495G or HGF β V495G mutants in phosphorylation- or peptide-dependent binding of the scHGF β-chain to Met provides additional data indicating that peptide activators require an accessible activation pocket in scHGF. However, the V495G mutants are less responsive to peptide activation than the scHGF mutants. This is probably due to adverse steric effects of the cleaved V495G terminal peptide bond, resulting in insertion of the positively charged and hydrophobic N-terminal Val (blue triangle, white plus sign) into the β-chain activation pocket (AP) and rearrangement of the pseudoactive site for productive Met binding (Mp). Met signaling requires HGF/Met dimerization by an as yet undetermined mechanism. In the case of peptide activators (bottom pathway), the N terminus of a peptide mimicking the β-chain N terminus (e.g., peptide V8) reversibly inserts into the β-chain activation pocket of pro-HGF and shifts the β-chain into an activated state capable of productive binding to Met. The peptide-bound form of pro-HGF is capable of Met signaling in the absence of proteolytic processing, presumably by a dimerization mechanism similar to that described above.

Our data herein support an alternative non-proteolytic allosteric activation mechanism (Fig. 6, bottom pathway). Here, peptide V8 can bind pro-HGF by inserting its N terminus into the activation pocket of the zymogen-like form of the β-chain, resulting in a conformational change to an active-like state at the Met binding site (Mn). The V8-bound pro-HGF active-like conformation is similar to that of two-chain HGF observed in the activation cleavage pathway and can productively bind to Met, ultimately resulting in signal transduction. The entire pathway must include a dimerization mechanism, which is still poorly understood at the molecular level.

Comparison with Other Trypsin-like Serine Protease Activation Pockets—The peptide activation mechanism for pro-HGF is akin to the molecular sexuality hypothesis initially proposed for the nonproteolytic activation of plasminogen by streptokinase (32). Despite the fact that the HGF β-chain is a non-catalytic serine protease, it can also utilize this mechanism to specifically regulate the pseudoactive site for Met binding by the HGF β-chain and signaling by full-length HGF. This mechanism has also been referred to as the “N-terminal insertion hypothesis” (44), which describes the allosteric regulatory process involving the intermolecular regulation of several trypsin-like serine proteases by pathogenic cofactors (45–48). Various strains of the bacterial pathogens Streptococci and Staphylococcus utilize this molecular mechanism by secreting activators of host zymogen serine proteases that bypass the requirement for cleavage. Streptokinase stimulates the catalytic activity of plasminogen (45, 49, 50), whereas staphylocoagulase and Von Willebrand factor-binding protein do so for prothrombin (46–48). These bacterial proteins bind to their respective zymogens and insert a surrogate N terminus into their activation pockets, providing examples of an intermolecular form of the serine protease activation mechanism. Complexes of staphylocoagulase bound to prothrombin-2 or α-thrombin provide strong structural support for this mechanism (46).

Short peptides that mimic the N terminus of streptokinase, which inserts its N terminus into the activation pocket of plasminogen, can induce proteolytic activity of a complex between plasminogen and an N-terminal deletion mutant of streptokinase (45). In this study, plasminogen activation was
Peptide Activators of Pro-HGF Stimulate Met Signaling

positively correlated with increasing peptide length, where even the first two residues displayed weak activity. However, peptides added in the absence of the streptokinase mutant were unable to activate plasminogen by themselves (45). In our study, short HGF β-chain N-terminal peptides (V2–V6) were unable to activate pro-HGF. It was not until we tested longer peptides (V7–V10) that we observed peptide-dependent “activation” of Met signaling by pro-HGF. Thus, there is a specific length dependence for peptide activation that may involve specific contacts made by pro-HGF and peptide residues at these extended positions that result in a signaling-competent state.

For trypsin-like serine protease zymogens, the N-terminal insertion mechanism is thermodynamically coupled to the conformation of the active site and an important component of their structural plasticity. Protease-like conformations of zymogens are typically highly favored only after activation cleavage or upon complexation with protease inhibitors, such as bovine pancreatic trypsin inhibitor (35–37, 51–53). Insertion of peptides into the zymogen activation pocket thermodynamically also favors a protease-like conformation for binding active site inhibitors; by microscopic reversibility, interactions by active site inhibitors that stabilize the protease-like conformation also favor binding of peptides to the zymogen activation pocket (28, 34–38, 52, 53). Thermodynamic coupling between peptide insertion into the pro-HGF β-chain activation pocket and Met binding to the pseudoactive site has not been formally addressed, but we suspect a similar linkage between these two binding events. However, there are key differences in the active site regions of bona fide serine proteases and HGF, such as the Trp at residue 215 found in almost all serine proteases, which is a Pro in HGF such as the Trp at residue 215 found in almost all serine proteases, which is a Pro in HGF (57–59, 61, 63, 64), the HGF β-chain in each dimer are highlighted as sticks in green or blue. The antiparallel nature of the N-terminal residues is apparent from the numbering of these residues. Asp353 (194 in chymotrypsin numbering) is shown as sticks and forms a salt bridge with the positively charged N terminus of Val495 (16 in chymotrypsin numbering). B, the first 4 residues of N terminus and Asp353 from one of the HGF β monomers are shown as sticks, and the other monomer is shown as a surface. The first 4 residues of each HGF β monomer are colored green or blue. C, same as B except that the first 8 residues of each HGF β monomer are shown. It is important to note that although there is no structure of the pro-form of HGF β, it must have an exposed loop accessible to proteases, such that it permits peptide V8 added in trans to bind to the β-chain monomer of pro-HGF in a manner somewhat related to that observed for the activated form of HGF. Images were made using PyMOL (72).

Mechanistic Implications for HGF-dependent Met Signaling—Although multiple models have been suggested regarding the stoichiometry of the signaling complex, current data from analytical ultracentrifugation, small angle x-ray scattering, cryo-electron microscopy, and structural biology best support a 2:2 signaling complex of HGF-Met where heparin may also play a significant role (29–31, 57–61). The high affinity site for Met resides on the α-chain, primarily due to N-terminal and K1 domain interactions (62), whereas the low affinity site is on the β-chain (29, 62). Dimerization mechanisms have been proposed to occur through the NK1-NK1 interface of HGF (57–59, 61, 63, 64), the HGF β-HGF β interface (29, 30), and via Met-Met interactions of the Sema domain (65).

It is striking that the N-terminal peptide of the HGF β-chain must have a minimum of 7 residues to activate pro-HGF for Met signaling. Prior studies exploring the molecular sexuality hypothesis have shown that N-terminal dipeptides are sufficient for activation (32, 38, 45). Why does pro-HGF require longer peptides for activation? We hypothesized two explanations for this phenomenon. The first and simpler one is that longer peptides have increased binding affinity. The second and more mechanistically interesting possibility involves the putative location of these peptides in the signaling complex. In the crystal structures of both HGF β alone and in complex with the Sema/PSI domain of Met, we observed the same HGF β-HGF β dimer (29, 30), which involves the newly cleaved N terminus precisely located at the dimer interface (Fig. 7). Although no structure of the pro-form of HGF β (i.e. scHGF β) exists, the residues at the dimer interface must be accessible for proteolytic cleavage, and thus their location must differ from those in the active form shown in Fig. 7 in order to permit peptide V8 binding when added in trans. This is notable because this β-β dimer offers a simple biochemical explanation for the requirement of activation cleavage of pro-HGF for signaling competency. The longer peptides may be...
sufficient to facilitate a similar dimerization and activate pro-HGF for Met signaling, whereas the shorter peptides are too short to span this dimer interface (Fig. 7, B and C). Our data imply that the longer peptide V8 binds with higher affinity than the shorter peptide V4, which favors the simpler explanation but also does not rule out the dimer interface proposal. Attempts to gain evidence for a HGF β-HGF β dimer in solution have proven unsuccessful, implying that if such a dimer exists, the affinity is relatively weak. However, because the proteins are in close proximity due to Met binding at the high affinity HGF α-chain and low affinity β-chain, there is no need for a high affinity β-β interaction.

Implications for HGF Signaling and Reversible Activators of Pro-HGF—Significant emphasis has been placed on the inhibition of HGF/Met signaling, given its role in driving tumor formation in various cancers (3, 11, 12). However, activation of HGF signaling is also very important for tissue regeneration and wound healing (10, 66). Importantly, in cell-based assays, we demonstrate that the V8 peptide-bound form of scHGF is capable of stimulating cell survival and migration to essentially the same extent as that stimulated with proteolytically cleaved two-chain HGF ligand (Fig. 5, A and B). As found with Met phosphorylation and binding data, the activation pocket is also critical for cellular signaling based on the lack of activity for peptide G8 or HGF D672N (Fig. 5A). It is not surprising to see a more robust effect in downstream cellular signaling events compared with the more modest upstream phosphorylation of Met.

In the absence of upstream proteolytic activation, these peptides provide the basis for an alternative reversible mechanism to stimulate HGF-dependent Met signaling. In vivo proteolytic activation of pro-HGF is irreversible, may be rate-limiting as well as tissue-specific, and is also highly regulated by inhibitors of proteases that activate pro-HGF (18, 67, 68). Recent data suggest that increased HGF activity is beneficial for resolving chronic wound disorders (69). However, given the oncogenic nature of HGF signaling, it may be useful to devise novel strategies, such as allosteric activators, for reversible activation of pro-HGF in order to better control Met signaling for different therapeutic applications. Further biochemical and structural characterizations of the peptide-bound form of pro-HGF should provide a better understanding of the allosteric activation mechanism and possibly yield insight into the design of more potent activators that target the HGF β-chain.

There is great interest in allosteric activators of signaling enzymes as well as receptor ligands (70). Recently, novel small molecule allosteric activators of procaspase-3 and -6, of the cysteine protease family, were discovered that act by shifting the equilibrium of the zymogen toward the active state, resulting in autoproteolytic caspase activation (71). Although HGF is not an enzyme, we have provided a new example of how allosteric activators of thezymogen-like form of the ligand can be used to drive receptor tyrosine kinase signaling pathways. It will be interesting to determine the general applicability of this approach to designing other peptide-based allosteric activators ofzymogen serine proteases and pseudoproteases.

Acknowledgments—We acknowledge Terry Lipari for purification of HGF proteins and Jeff Tom for purification of peptides. We thank Meredith Sagolla and the Center for Advanced Light Microscopy for help with the cell-based scratch assays. We also thank Genentech support laboratories for oligonucleotide synthesis, DNA sequencing, and peptide synthesis and purification. Finally, we acknowledge Erin Dueber and Andrea Cochran for critical reading of the manuscript.

REFERENCES

1. Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimoniishi, M., Sugimura, A., Tashiro, K., and Shimizu, S. (1989) Nature 342, 440–443
2. Bussolino, F., Di Renzo, M. F., Ziche, M., Bocchietto, E., Olivero, M., Naldini, L., Gaudino, G., Tamagnone, L., Coffier, A., and Comoglio, P. M. (1992) J. Cell Biol. 119, 629–641
3. Birchmeier, C., Birchmeier, W., Gherardi, E., and Vande Woude, G. F. (2003) Nat. Rev. Mol. Cell. Biol. 4, 915–925
4. Stoker, M., Gherardi, E., Peryman, M., and Gray, J. (1987) Nature 327, 239–242
5. Derman, M. P., Cunha, M. J., Barros, E. J., Nigam, S. K., and Cantley, L. G. (1995) Ann. J. Physiol. 268, F1211–F1217
6. Webb, C. P., Taylor, G. A., Jeffers, M., Fiscella, M., Oskarsson, M., Re- sau, J. H., and Vande Woude, G. F. (1998) Oncogene 17, 2019–2025
7. Bottaro, D. P., Rubin, J. S., Faletto, D. L., Chan, A. M., Kmieciek, T. E., Vande Woude, G. F., and Aaronson, S. A. (1991) Science 251, 802–804
8. Grant, D. S., Kleinman, H. K., Goldberg, I. D., Bhargava, M. M., Nick- oloff, B. J., Kinsella, J. L., Polverini, P., and Rosen, E. M. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 1937–1941
9. Watanabe, S., Hirose, M., Wang, X. E., Maehiro, K., Murai, T., Kobayashi, O., Nagahara, A., and Sato, N. (1994) Biochem. Biophys. Res. Com- mun. 199, 1453–1460
10. Bevan, D., Gherardi, E., Fan, T. P., Edwards, D., and Warn, R. (2004) J. Pathol. 203, 831–838
11. Mauk, G., Shrikhande, A., Kijima, T., Ma, P. C., Morrison, P. T., and Salgia, R. (2002) Cytokine Growth Factor Rev. 13, 41–59
12. Mazzone, M., and Comoglio, P. M. (2006) FASEB J. 20, 1611–1621
13. Xin, X., Yang, S., Ingle, G., Zlot, C., Rangell, L., Kowalski, J., Schwall, R., Ferrara, N., and Gerritsen, M. E. (2001) Am. J. Pathol. 158, 1111–1120
14. Conway, K., Price, P., Harding, K. G., and Jiang, W. G. (2006) Wound Repair Regen. 14, 2–10
15. Nakamura, T., and Mizuno, S. (2010) Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 86, 588–610
16. Eder, J. P., Vande Woude, G. F., Boerner, S. A., and LoRusso, P. M. (2009) Clin. Cancer Res. 15, 2207–2214
17. Comoglio, P. M., Giordano, S., and Trusolino, L. (2008) Nat. Rev. Drug Discov. 7, 504–516
18. Kataoka, H., and Kawaguchi, M. (2010) FEBS Lett. 577, 2230–2237
19. Naldini, L., Tamagnone, L., Vigna, E., Sachs, M., Hartmann, G., Birch- meier, W., Daikuaha, Y., Tsuzuchi, H., Blasi, F., and Comoglio, P. M. (1992) EMBO J. 11, 4825–4833
20. Shimomura, T., Miyazawa, K., Komiyama, Y., Hiraoka, H., Naka, D., Morimoto, Y., and Kitamura, A. (1995) Eur. J. Biochem. 229, 257–261
21. Lee, S. L., Dickson, R. B., and Lin, C. Y. (2000) J. Biol. Chem. 275, 36720–36725
22. Peek, M., Moran, P., Mendoza, N., Wickrasmasinghe, D., and Kirchhofer, D. (2002) J. Biol. Chem. 277, 47804–47809
23. Donate, L. E., Gherardi, E., Srinivasan, N., Sowdhamini, R., Aparicio, S., and Blundell, T. L. (1994) Protein Sci. 3, 2378–2394
24. Tordai, H., Banay, L., and Pothy, L. (1999) FEBS Lett. 461, 63–67
25. Hartmann, G., Naldini, L., Weidner, K. M., Sachs, M., Vigna, E., Como- glio, P. M., and Birchmeier, W. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 11574–11578
26. Lokker, N. A., Mark, M. R., Luis, E. A., Bennett, G. L., Robbins, K. A., Baker, J. B., and Godowski, P. J. (1992) EMBO J. 11, 2503–2510
27. Huber, R., and Bode, W. (1978) Acc. Chem. Res. 11, 114–122
