Structural basis for agonism and antagonism of hepatocyte growth factor

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Hepatocyte growth factor (HGF) is an activating ligand of the Met receptor tyrosine kinase, whose activity is essential for normal tissue development and organ regeneration but abnormal activation of Met has been implicated in growth, invasion, and metastasis of many types of solid tumors. HGF has two natural splice variants, NK1 and NK2, which contain the N-terminal domain (N) and the first kringle (K1) or the first two kringle domains of HGF. NK1, which is a Met agonist, forms a head-to-tail dimer complex in crystal structures and mutations in the NK1 dimer interface convert NK1 to a Met antagonist. In contrast, NK2 is a Met antagonist, capable of inhibiting HGF's activity in cell proliferation without clear mechanism. Here we report the crystal structure of NK2, which forms a "closed" monomeric conformation through interdomain interactions between the N- domain and the second kringle domain (K2). Mutations that were designed to open up the NK2 closed conformation by disrupting the N/K2 interface convert NK2 from a Met antagonist to an agonist. Remarkably, this mutated NK2 agonist can be converted back to an antagonist by a mutation that disrupts the NK1/NK1 dimer interface. These results reveal the molecular determinants that regulate the agonist/antagonist properties of HGF NK2 and provide critical insights into the dimerization mechanism that regulates the Met receptor activation by HGF.

cancer metastasis | crystal structure | HGF agonist | HGF antagonist | Met receptor tyrosine kinase

Hepatocyte growth factor (HGF) and the Met tyrosine kinase receptor form a unique ligand-receptor signaling system where HGF is the only known endogenous ligand that activates Met. HGF is also known as a scattering factor, and its signaling through Met activation is involved in promoting cell proliferation, survival, migration, and branching in a variety of cell types with important roles in angiogenesis, wound repair, and cell migration during development (1, 2). Inappropriate expression of Met or HGF has been implicated in many cancers and is associated with an aggressive phenotype and poor prognosis (1, 3) (www.vai.org/met). Thus, inhibition of Met activity, either by small molecule kinase inhibitors or by protein-based HGF antagonists, has become an important and rational strategy for developing anticancer therapeutics.

The Met extracellular domain (ECD), which encompasses approximately 900 amino acids (25–932), is cleaved into α and β chains by the furin protease between residues 307 and 308. The first 519 amino acids of Met form a 7-bladed β-propeller domain, called sema domain, with homology to the semaphorin and plexin protein families (4, 5). Following the sema domain are a cysteine-rich domain and four immunoglobulin-like domains. The sema domain of Met is necessary and sufficient for binding and activation of the receptor by HGF (6). It is believed that HGF induced Met activation is mediated through a formation of a 2:2 complex where Met dimerization is primarily mediated by dimer formation of HGF (7, 8).

HGF is a disulfide-linked two chain protein of 728 amino acids with its α-chain containing an N-terminal domain and four kringle domains, and its β chain containing a serine protease domain. HGF belongs to the family of serine protease growth factors, and is most similar to plasminogen and macrophage stimulating factor (MSP), which is the ligand for RON, a receptor closely related to Met. Unlike plasminogen, neither HGF nor MSP harbors protease activity as the key catalytic residues are missing in their protease domains. HGF is synthesized as a single chain proHGF, which binds strongly to Met but it is inactive (6). Activation of proHGF requires a cleavage at arginine-494, which induces conformational changes in the inactive serine protease β-chain, thus allowing it to bind to the sema domain of Met (9). The α-chain of HGF also binds to the Met sema domain independently of the β-chain (10). Cleavage of HGF before cysteine 487 occurs with mast cell chymase, plasma kallikrein, or neutrophil elastase, each of which generates a free α-chain that can function as a competitive inhibitor of HGF (2, 11). A synthetic version of the HGF α-chain containing the N-terminal domain and four kringle domains (NK4) has been explored as a HGF antagonist for anticancer therapy (12–15). Full-length HGF antagonists can also be generated by mutating arginine-494 to glutamate, which makes a noncleavable proHGF, or by mutations in the "activation pocket" of the HGF β-chain (7, 16).

HGF has two naturally occurring splice variants. The first, NK1, acts as a Met agonist, but requires the presence of heparan sulfate for full activity (17–19). NK1 contains a portion of the full HGF α-chain that includes the N terminus and the first kringle domains. Crystal structures of both mouse and human NK1 reveal a head-to-tail dimer configuration, and this NK1 dimer interface is required for agonist activity of NK1 and of full-length HGF itself. Mutations in the residues of the NK1 dimer interface convert NK1 into a competitive Met antagonist (8, 20). A second splice variant, NK2, consisting of the N-terminal domain and first two kringle domains, was originally described as a competitive HGF antagonist (21). NK2 lacks the ability to activate Met and specifically inhibits HGF-mediated cell proliferation (22, 23). The mechanism of how NK2 inhibits Met activation remains unclear. In this paper, we describe the crystal structure of the human NK2, which exists as a monomer with a closed conformation that is tethered by intramolecular interactions between the N-domain and the K2 domain. This closed conformation prevents NK2 from forming the dimer via the established NK1/NK1 interface. We show that mutations that disrupt this conformation allow NK2 to promote Met dimerization via the NK1/NK1 dimer interface, thereby converting NK2 from a Met antagonist to an agonist.

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Data deposition: The structure coordinates and diffraction data have been deposited in the Protein Data Bank, www.pdb.org [PDB ID codes 3HN4 (human NK2), 3HMR (mouse N-domain), 3HMT (human N-domain dimer), and 3HMS (human N-domain monomer)].

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Furthermore, this mutated NK2 agonist can be changed back to a Met antagonist by mutations that disrupt the NK1/NK1 dimer interface. Thus our results provide a mechanistic explanation for NK2 antagonist activity and a rational basis for the inherent properties that distinguished between NK1/NK2 agonist/antagonist properties of HGF.

Results

Direct Binding of NK2 and Other HGF Derivatives to the Met Receptor ECD. To investigate the interactions of NK2 and other HGF derivatives with Met, we purified all protein components for binding assays. The Met ECD (residues 25–567), which contains the sema and cysteine-rich domains, was purified as previously reported (5). The NK2 (residues 28–289) contains three mutations: K132E, R134E, and C214A. The K132E and R134E mutations, which were first made in NK1(1K1) by Lietha, et al. (18), not only keep the agonist activity of NK1 but also protect the protein from proteolysis during purification. C214 is predicted to form a disulfide bond with C345 from the kringle-3 domain in longer versions of HGF, and its mutation to alanine in NK2 is to prevent nonspecific intermolecular disulfide bond formation (24). The binding of NK2 to Met was measured by its ability to compete with biotinylated NK1, which interacted directly with Met in protein-protein interaction assays (8). NK2 binds to Met with a similar IC50 of 335 nM and 280 nM in the absence or presence of heparin, suggesting NK2 can effectively interact with Met independent of heparin (Fig. 1*A*, *B*). NK2's heparin independence is in contrast to NK1, which binding to Met is largely dependent on heparin, with IC_{50} values of 8.5 ± 4.6 μM in the absence of heparin and 10.4 ± 1.1 nM in the presence of heparin, increasing NK1 binding affinity to Met by >800 fold (Fig. 1 *A*, *B* and Table 1).

To examine the role of heparin in NK1 binding and activation of Met, we made mutations in heparin binding residues of human NK1 (R73E, K58A/K60A/T61A/K62A, K58E/K60E/T61A/K62E, and K58E/K60E/T61A/K62E/R73E). Some of these mutations are similar to those made by Lietha et al. (18). These NK1 mutants were made with the additional R134G mutation to aid in thioredoxin tag removal and purification (8). Removal or charge reversal of heparin binding residues within the N-terminal domain allowed the binding of NK1 to Met independent of heparin (Fig. 1 *C*, *D*). Interestingly, removing the positive charges at K58, K60, and K62 by mutation to alanine generated a mutant with a slightly higher affinity to Met than reversing the charges by mutation to glutamate acid. Reversing the single charged residue R73 to a glutamate acid also produced a mutant that bound to Met independent of heparin. These results suggest that the primary role of heparin in NK1 binding to Met is to neutralize the positive charged residues on the N-domain surface. In the case of NK2, the presence of the second kringle domain replaces much of the requirement of heparin for higher affinity binding (Fig. 1 *A*, *B*).

Biochemical Characterization of NK2 as a Met Antagonist. NK2 was shown previously to inhibit HGF-mediated Met activation in cell-based assays. To assess whether the purified NK2 protein also functions as a Met antagonist, we performed protease assays for the urokinase-type plasminogen activator (uPA) because it is induced by Met activation in Madin-Darby canine kidney (MDCK) cells. Upon HGF treatment, uPA activity was elevated to 3–5-fold in MDCK cells (Fig. 2*A*). Met activation by HGF also induced scattering of MDCK cells (Fig. 2*B*). NK2 itself failed to activate uPA regardless of the presence or absence of heparin (Fig. 2*A*). Furthermore, NK2 inhibited HGF-mediated uPA activation and scattering of MDCK cells (Fig. 2 *B*, *C*). The activity of NK2 is similar to the Y124A mutated NK1, which is a Met antagonist with a defective mutation in its dimer interface. Although the biochemical mechanisms of NK2 as a Met antagonist remain unclear, by analogy to NK1 (Y124A) antagonist, we reasoned that NK2 can bind to Met but is unable to induce Met dimerization. Indeed, NK2 failed to induce dimerization of the purified Met ECD, similar to the NK1 (Y124A) antagonist (Fig. 2*D*). In contrast, wild-type NK1, a Met agonist, readily promoted Met dimerization in the presence of heparin (Fig. 2*D*). Together, these results establish that NK2 serves as a Met antagonist through binding to Met and inhibition of Met dimerization.

Structural Basis for NK2 as a Met Antagonist. The human NK2 was crystallized in space group $p6_522$ with one molecule per asymmetric unit. The structure was solved by molecular replacement with the N-domain and kringle domain from NK1 as searching models. The structure was refined to an *R*-factor of 26.3% and *R*-free of 33.3% at a resolution of 2.6 Å. For structural comparisons, we also determined the crystal structure of the isolated N-domain from human and mouse at resolution ranges of 1.7 Å–2.0 Å (Fig. S1). The statistics of the dataset and refinement are summarized in Table S1.

The overall structure of NK2 adopts a "closed" triangle conformation with the two kringle domains sitting at the bottom and the N-domain at the top (Fig. 3*A*). Within this triangle conformation, the N-domain structure is nearly identical to the N-domain structure in an isolated form or in the NK1 form (Fig. 3*B*). The

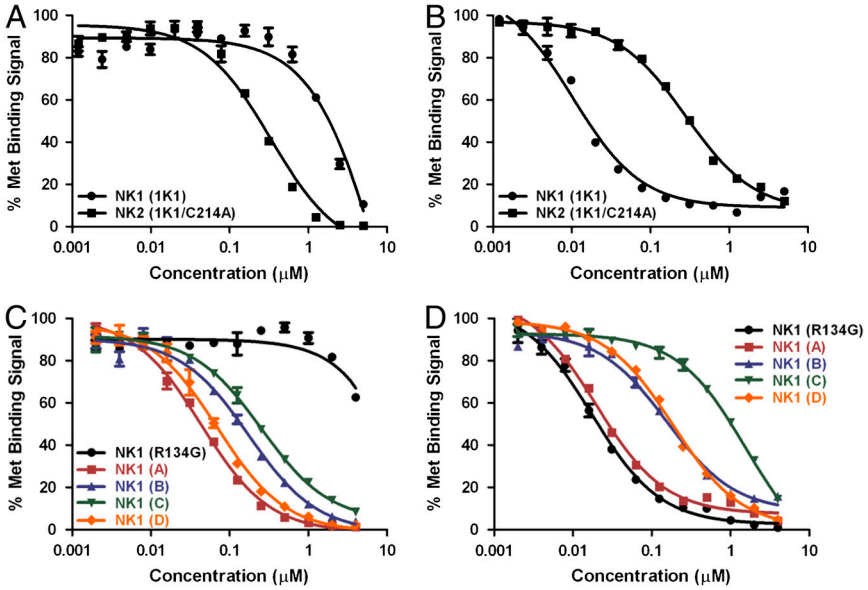

Fig. 1. Heparin independent binding of NK2 to Met (*A* and *B*). Binding of NK1 and NK2 to Met, as determined by competition of the binding of biotinylated NK1 to Met in AlphaScreen Assays, in the absence (*A*) and presence (*B*) of heparin. Both NK1 and NK2 contain the K132E and R134E mutations that were designated as 1K1 (18). (*C* and *D*). Heparin independent binding of NK1 mutants to Met, as determined by AlphaScreen competition curves, for the Met567-NK1 interaction in the absence (*A*) and presence (*B*) of heparin. The NK1 mutants tested are: NK1(R134G) (*black*), NK1(A)—NK1(R134G,R73E) (*red*), NK1(B)—NK1(R134G,K58A,K60A,T61A,K62A) (*blue*), NK1(C)—NK1(R134G,K58E,K60E,T61A,K62E) (*green*), and NK1(D)—NK1(R134G,K58E,K60E,T61A, K62E,R73E) (*orange*). IC50 values derives from these curves are summarized in Table 1.

Table 1. IC_{50} values for NK1 (1K1) and NK2 mutants in the presence and absence of heparin using biotinylated NK1 and histidine tagged Met567 in an AlphaScreen competition assay.

NK2 mutants	IC_{50} (nM) (– heparin)	IC_{50} (nM) (+ heparin)
NK1 (1K1)	$8.5 \times 10^3 \pm 4.6 \times 10^3$	10.4 ± 1.1
1K1/C214A	335 ± 44	281 ± 20
1K1/C214A/**D68R**	65.2 ± 9.6	44.3 ± 6.2
1K1/C214A/**D257A/N258A**	64.6 ± 7.4	2.85 ± 0.31
1K1/C214A/**D68R/D257A/N258A**	462 ± 103	65.1 ± 7.3
1K1/C214A/**Y124A/D257A/N258A**	704 ± 93	186 ± 29

1K1 represents the K132E and R134E mutations.

two kringle domains are also highly similar to each other (Fig. 3*C*). These results suggest that the N-domain and the two kringle domains have rigid structures with the major conformational changes occurring at the linker regions between the domains. Compared to the NK1 dimer structure (Fig. 3*D* and Fig. S2), NK2 adopts a monomeric configuration with its K2 domain displacing the K1 domain of the NK1 dimer structure. The K1 domain in the NK2 structure is rotated approximately 180° relative to its position in the NK1structure, into the space that would be occupied by the neighboring NK1 monomer in the dimeric NK1 structure. The rotation of the K1 domain in the NK2 structure is mediated by the flexible linker region between the N-terminal and K1 domains. Most of the rotation occurs between residues 122–127 of the linker region, which is the main interface of the NK1/NK1 dimer. The rotation of the K1 domain in the NK2 structure prevents NK2 from adopting a dimer configuration, therefore providing a structural basis for NK2 antagonism.

N-Terminal Domain/K2 Interface. The "closed" conformation in the NK2 crystal structure is held together by a series of charged and hydrogen bond interactions between the N- and K2 domains, specifically K122-D224, K122-N258, D68-H237, and K44-D236. D236 and H237 are in turn stabilized by R234 (Fig. 3*E*, Table S2). D257 forms a hydrogen bond with the main chain nitrogen of K44 as well as helping to stabilize R234 from the K2 domain. In this closed conformation, many of the NK2 residues reported to be involved in Met binding remain accessible (25, 26). Key Met-binding residues E159, S161, E195, R197, and Y198 are placed outward on the K1 domain along with N-terminal domain heparan sulfate binding residues K58, K60, T61, K62, R73, and R76 (18). In this arrangement, the K2 domain in the NK2 structure would inhibit dimer formation in the Met/NK2 complex and prevent Met activation. Thus, NK2 is able to bind to but not activate Met, consistent with its antagonism property.

To generate the NK1 dimer with NK2 one would need to rotate the first kringle domain about the linker region and then move the second kringle domain into a more "open" conformation. In the NK2 crystal structure, intramolecular interactions between the N-terminal domain and the K2 domain stabilize the closed conformation and sterically hinder the intermolecular contacts seen in the NK1 dimer interface. The linker region between the N- and K1 domains is highly flexible, as demonstrated by its conformational difference between the NK1 and NK2 structures. Linker flexibility suggests that NK2 could also adopt an open conformation and form a NK1-like dimer by disrupting the N/K2 interface. We show below by mutagenesis that such NK2 dimers may serve as a Met agonist.

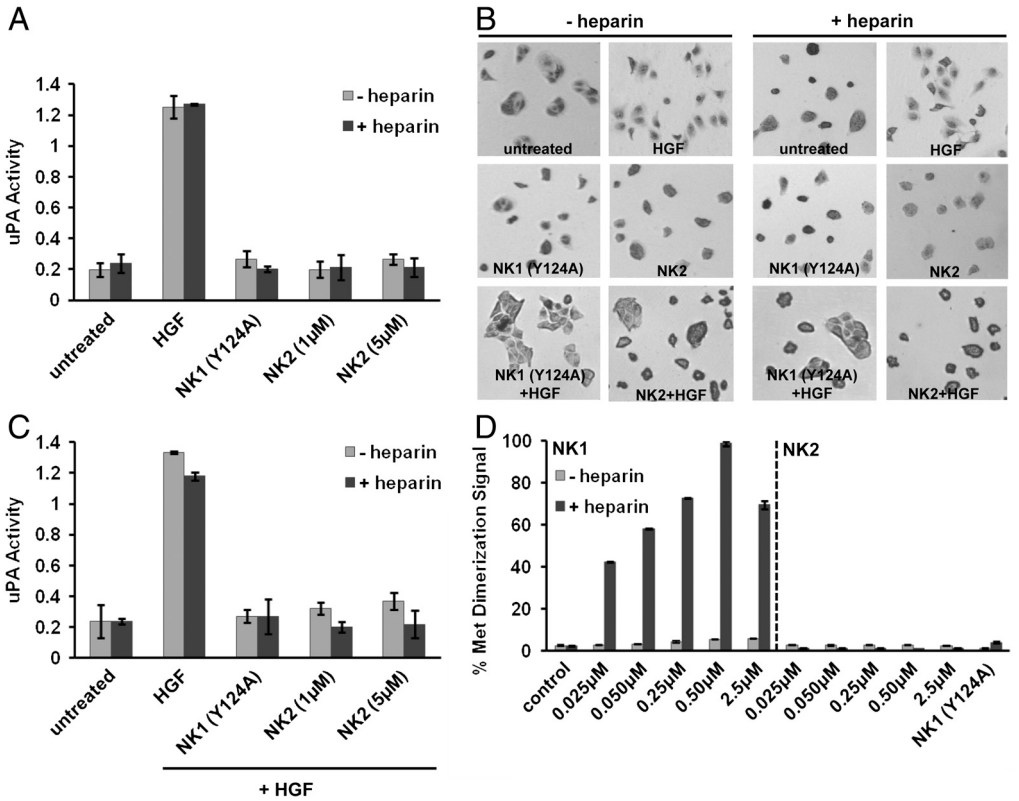

Fig. 2. NK2 is a Met antagonist (*A*) uPA induction of MDCK cells by HGF (60 ng/mL), the NK1(Y124A) antagonist, and NK2. (*B*) MDCK cell scatter assays for HGF (60 ng/mL), NK1(Y124A) (1 μM), NK2 (0.0125 μM), or HGF + NK1 (Y124A), and HGF + NK2, in the presence or absence of heparin. (*C*) Inhibition of HGF-mediated uPA activation by NK2 and the NK1(Y124A) antagonist. Experiments are identical to (*A*) except that 60 ng/mL HGF was added to wells that were pretreated with NK1(Y124A) or NK2. (*D*) NK2 fails to induce Met dimerization regardless of the presence of heparin. NK1 and NK1 (Y124A) are used as positive and negative controls for their ability to induce Met-dimerization, which is assayed based on a modified AlphaScreen reported previously (8). Values are normalized to the dimerization signal of NK1 at 0.50 μM in the presence of heparin.

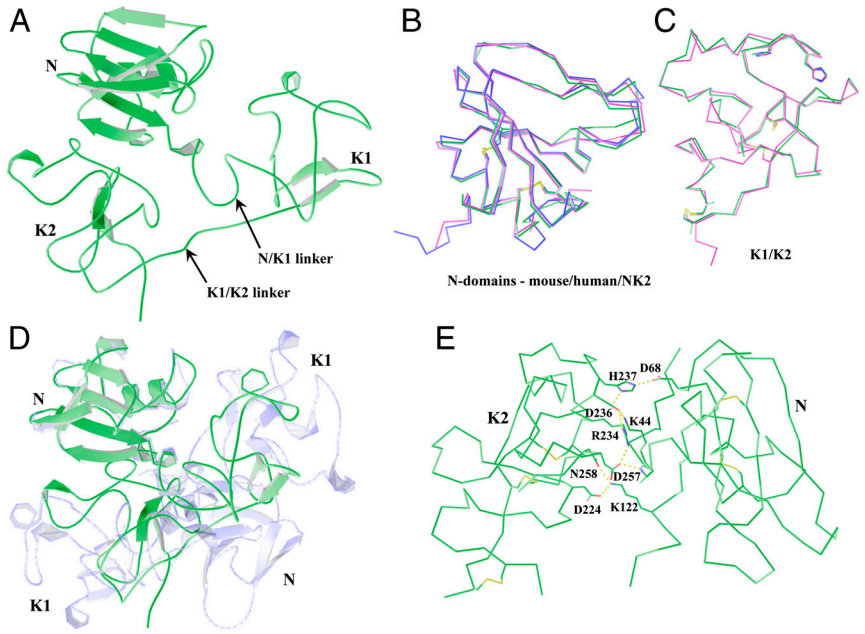

Fig. 3. Crystal structure of NK2 (*A*) NK2 is a monomer. The overall structure of NK2 is represented by a ribbon diagram. Individual domains are designated by N for the N-terminal domain, K1 for the first kringle domain, and K2 for the second kringle domain. (*B*) The N-domain has a rigid structure. An overlay of the mouse (*purple*) and human (*blue*) N-domain crystal structures with the N-domain from the NK2 structure (*green*) shows the rigid fold of the N-domain. K1 and K2 domains of NK2 are omitted for clarity. (*C*) Kringle domain has a conserved rigid structure as shown by an overlay of K1 (*purple*) and K2 (*green*) domains of NK2. (*D*) The dimeric structure of NK1 (PDB ID 2QJ2) (*blue*) is presented for direct comparison of the monomeric NK2 structure (*green*) represented in (*A*). (*E*) The N/K2 interface. The interactions between intramolecular N and K2 domains are are shown as *dashed yellow lines* and disulfide bonds are shown as *solid yellow lines*. Nitrogen and oxygen atoms in side chain or main chain atoms are colored blue or red respectively. Only the alpha carbon residues are shown for the residues not involved in either interface. The first kringle domain of the N/K2 interface is omitted for clarity. All structure figures were made with Bobscript (33–35) and Raster3D (36).

NK2 Mutations That Induce Met Dimerization and Activation. The closed conformation of the NK2 monomer is held by interactions between the N- and K2 domains. We have demonstrated the ability to interchange the agonist/antagonist property of NK1 by manipulating the NK1 dimer interface (8). By analogy to NK1 experiments, we predicted that disruption of the N/K2 interface may release the closed conformation of NK2 to a more open conformation like NK1, thus allowing NK2 to form a dimer complex with NK1 at the dimer interface and converting NK2 from a Met antagonist to an agonist.

To test this prediction, we made three NK2 mutants with mutations of D68R, D257A/N258A, and D68R/D257A/N258A, which were designed to disrupt the N/K2 interface. In contrast to the parent NK2 (Fig. 2*D*), these three NK2 mutants were able to induce Met dimerization in the presence of heparin (Fig. 4*A*), especially the D257A/N258A mutant, which promoted Met dimerization at concentrations as low as 25 nM (Fig. 4*A*). Similarly, all three NK2 mutants were able to activate uPA activity (Fig. 4*B*). In addition, the D257A /N258A NK2 mutant has the same ability as HGF in promoting the scattering of MDCK cells (Fig. 4*C*). These results demonstrate that NK2 can be converted from a Met antagonist to an agonist by the mutations in the N/K2 interface, which opens the closed conformation of NK2. The D257A/N258A NK2 mutant binds to Met potently with an estimated

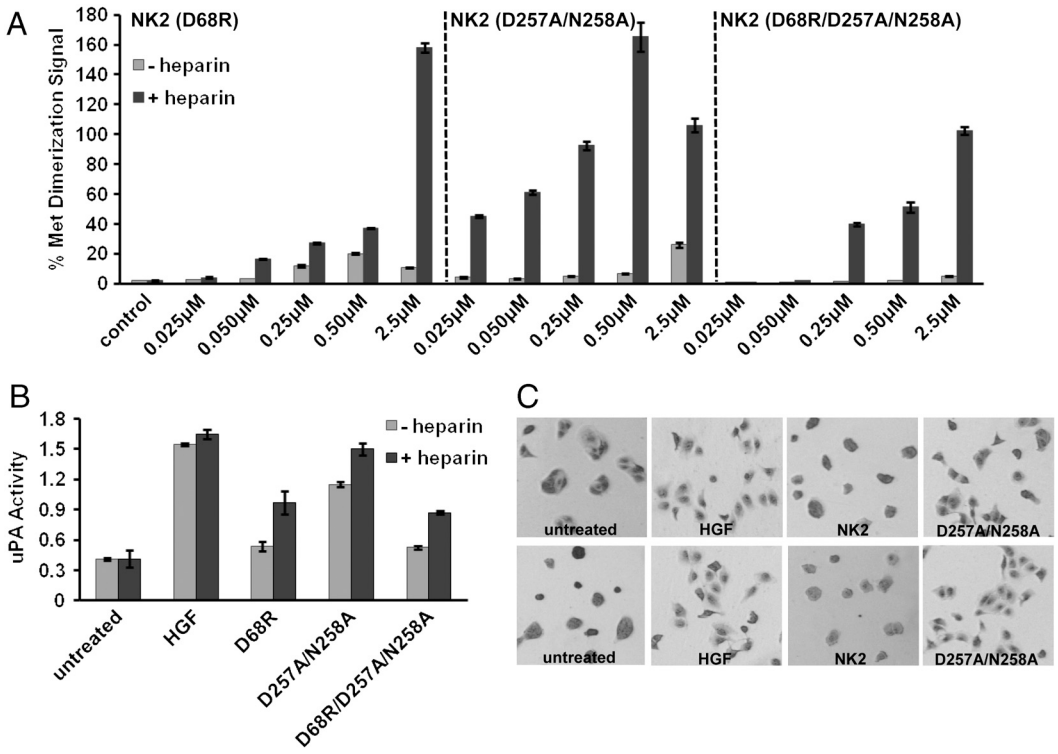

Fig. 4. Conversion of NK2 to a Met agonist by disrupting the N/K2 interface (*A*) Mutations that disrupt the N/K2 interface promote the ability of NK2 to induce Met dimerization, which is assayed based on a modified AlphaScreen reported previously (8). Three different mutated NK2 are shown in the presence and absence of heparin. (*B*) Induction of uPA by HGF (60 ng/mL) and the three NK2 mutated variants (0.0125 μM) in the presence and absence of heparin in MDCK cells. The D257A/N258A NK2 has nearly the same ability to induce uPA activation as HGF. (*C*) Scattering of MDCK cells by the D257A/N258A NK2 mutant (0.0125 μM) and HGF (60 ng/mL). Top row (-heparin), bottom row (+heparin).

affinity of 3 nM in the presence of heparin (Table 1). To determine whether the agonist property of these three NK2 mutants is mediated via an NK1-like dimer interface, we made the Y124A mutation in the NK1 dimer interface. Y124 is a key residue that forms the NK1 dimer interface and the Y124A mutation was previously designed to convert NK1 from a Met agonist to an antagonist (8). Similarly, the Y124A mutation converts the D257A/N258A NK2 mutant from a Met agonist to an antagonist as demonstrated by uPA activation and MDCK scattering assays. (Fig. 5*A*–*C*). Furthermore, as expected, the Y124A mutation abolished the ability of the D257A/N258A NK2 mutant to induce Met dimerization (Fig. 5*D*), suggesting that the NK2 agonist activity is also mediated through the NK1 dimer interface.

Discussion

It has been puzzling why NK1 and NK2, the two natural splice variants of HGF, have opposing effects on Met activation. We have previously reported the activation mechanism of Met by NK1, which forms a dimeric complex via an interface of the N/K1 linker region involving the Y124 residue (8, 20). In this paper, we have determined the structural and molecular mechanism of NK2 as a Met antagonist. We further explored this mechanism for converting NK2 from an antagonist into an agonist and then from an agonist to an antagonist. The overall mechanism of these conversions is based on the crystal structure of NK2, which reveals a monomeric complex with a closed conformation that is held by intramolecular interactions between the N- and K2 domains. The monomeric nature of NK2 is consistent with its activity as a Met antagonist because Met activation by HGF requires the dimerization of HGF, which is mediated by the NK1-NK1 dimer interface. The closed conformation of NK2 prevents it from forming an NK1-NK1 dimer configuration, thus preventing it from inducing Met dimerization and activation. On the other hand, the closed NK2 conformation remains capable of binding to Met given the full accessibility of the Met-binding residues in K1 domain. Therefore, NK2 is able to bind to Met but unable to activate it.

The closed conformation of NK1 can be opened up by introducing mutations that disrupt the N/K2 interface. Importantly, three of these NK2 mutants are able to activate Met with activity similar to NK1. The ability of these NK2 mutants to act as Met agonists suggests that NK2 has inherent Met activation activity, which is inhibited by the closed conformation of the N/K2 interface. Disruption of this interface opens up the NK2 conformation, which enables it to activate Met. Furthermore, these NK2 mutants activate Met through the same conserved mechanism of the NK1-NK1 dimer interface, as the Y124A mutation also abolishes the ability of these NK2 mutants to activate Met. The NK1-NK1 dimer interface has been shown to be required for Met activation by NK1 or by the full-length HGF (7, 8, 20). Our results here with NK2 further highlight the importance of the NK1-NK1 dimer interface in activation of the Met receptor. The above mechanistic understanding of Met activation by HGF has provided us a rational basis to manipulate HGF derivatives for therapeutic purposes as demonstrated by our ability to engineer NK2 from an antagonist to an agonist and then from an agonist to an antagonist.

Methods

Protein Production, Binding Assays, and Crystallization. Both mouse and human HGF NK2 (residues 28–289) was expressed as a 6xHis-thioredoxin fusion protein from the expression vector pET-Duet1 in the *Escherichia coli* strain Rosetta/gami(DE) (Novagen) to promote disulfide bond formation. The biotinylated proteins (NK1 and NK2) were produced by fusing the 20 amino acid biotin acceptor peptide sequence from the pDW464 plasmid (27) to the N terminus. The Met protein (residues 25–567, containing the sema domain and the cysteine-rich domain) was expressed as a C-terminal hexahistidine tag fusion protein from Lec 3.2.8.1 cells (28). All proteins were purified to homogeneity for binding assays and crystallization with details described in *SI Text*.

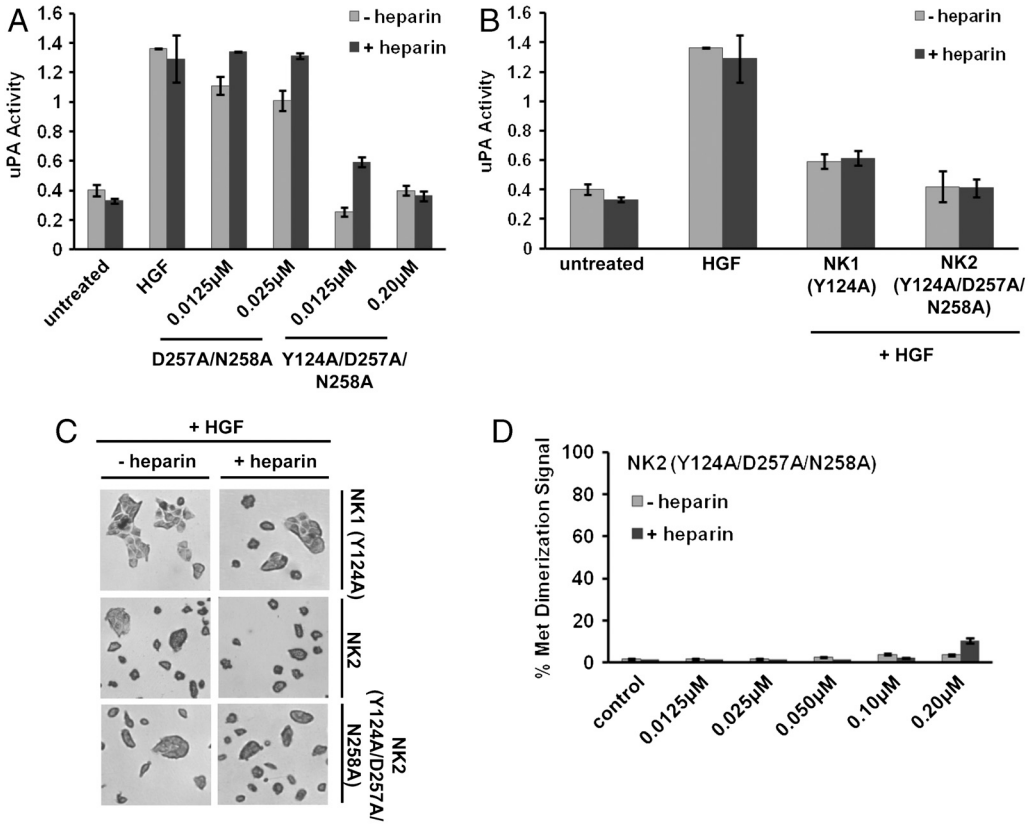

Fig. 5. Conversion of the D257A/N258A NK2 from a Met agonist to a Met antagonist by mutating Y124A in the NK1 interface. (*A*) uPA induction assays in MDCK cells for HGF (60 ng/mL), the D257A/N258A NK2, and the D257A/N258A NK2 containing the Y124A mutation in the NK1 interface show the Y124A mutation abolishes the ability of the D257A/N258A NK2 to activate uPA. (*B*) An antagonist assay shows the ability of Y124A mutated NK1 and NK2 (D257A/N258A) to inhibit HGF-mediated activation of uPA in MDCK cells. (*C*) An antagonist assay shows the ability of Y124A mutated NK1 and NK2 (D257A/N258A) to inhibit HGF-mediated scattering of MDCK cells. The Y124A NK1 and NK2 were used as antagonist controls. (*D*) The Y124A mutation abolishes the ability of the D257A/N258A NK2 to promote Met-dimerization as compared to Fig. 4*A*.

Data Collection and Structure Determination. Diffraction data were collected at 21-ID-D (Life Sciences (LS)-Collaborative Access Team (CAT)) of the Advance Photon Source with details described in *SI Text*. The structure was solved by molecular replacement using the Protein Data Bank (PDB) coordinates 1NK1 (29). Molecular replacement and model refinement were performed with Crystallography and NMR System (CNS), where twin fraction was incorporated for the refinement for the mouse structure, and manual model building was done with the program O (30). A Hepes and a sulfate molecule is found and modeled into the K1 and K2 domain (Fig. S3).

Met Activation Assays. Cell-based Met activation assays, including scattering of MDCK cells and uPA activation assays, followed published protocols (31, 32) with details described in *SI Text*.

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