Mutations in the met Oncogene Unveil a “Dual Switch” Mechanism Controlling Tyrosine Kinase Activity*

The met oncogene, encoding the high affinity hepatocyte growth factor receptor, is the only known gene inherited in human cancer that is invariably associated with somatic duplication of the mutant locus. Intriguingly, mutated Met requires ligand stimulation in order to unleash its transforming potential. Furthermore, individuals bearing a germ line met mutation develop cancer only late in life and with incomplete penetrance. To date, there is no molecular explanation for this unique behavior, which is unusual for a dominant oncogene. Here we investigate the molecular mechanisms underlying met oncogenic conversion by generating antibodies specific for the differently phosphorylated forms of the Met protein. Using these antibodies, we show that activation of wild-type Met is achieved through sequential phosphorylation of Tyr1234 and Tyr1235 in the activation loop and that mutagenesis of either tyrosine dramatically impairs kinase function. Surprisingly, oncogenic Met mutants never become phosphorylated on Tyr1234 despite their high enzymatic activity, and mutagenesis of Tyr1235 does not affect their biochemical or biological function. By analyzing the enzymatic properties of the mutant proteins in different conditions, we demonstrate that oncogenic mutations do not elicit constitutive kinase activation but simply overcome the requirement for the second phosphorylation step, thus reducing the threshold for activation. In the presence of activating signals, these mutations result in a dynamic imbalance toward the active conformation of the kinase. This explains why mutant met provides an oncogenic predisposition but needs a second activating “hit,” provided by sustained ligand stimulation or receptor overexpression, to achieve a fully transformed phenotype.

The Met tyrosine kinase, a high affinity receptor for hepatocyte growth factor (HGF),1 plays a pivotal role in controlling cell growth, motility, differentiation, and survival (1, 2). The fine tuning of these processes by Met is central for embryonic development, organ formation, wound healing, and tissue regeneration (3–6). Inappropriate activation of the Met pathway leads to the acquisition of transforming and invasive potential and is a recurrent event in some types of human cancer (7–9). Point mutations in the met proto-oncogene, corresponding to amino acid substitutions in the kinase domain of Met, co-segregate with hereditary papillary renal carcinomas (10) and have been described in sporadic tumors as well (10–13). Mutant Met has been shown to display deregulated kinase activity to transform mouse fibroblast and to be tumorigenic in nude mice (14, 15), although the molecular mechanism underlying this oncogenic conversion is still unclear. Interestingly, although the mutant forms of Met possess an intrinsic higher kinase activity in vitro, their transforming ability in vivo is latent and can be unmasked only in the presence of HGF (16). Patients harboring a germ line mutation in the met gene develop cancer only late in life and with incomplete penetrance (10), most probably because duplication of the mutant allele is required for tumor progression (17, 18). All together, these observations indicate that a met mutation provides tumor predisposition, but increased dosage and/or sustained ligand stimulation is required to actually determine tumor formation. To date, there is no molecular explanation for this unique biological behavior, which is unusual for a dominant oncogene.

From a biochemical viewpoint, receptor tyrosine kinases (RTKs) are nothing but allosteric enzymes that may exist in an inactive and an active conformation. The transition from the inactive to the active state is regulated by both cis-autoinhibition and trans-autophosphorylation. In the inactive form, access of substrate or substrate and ATP to the catalytic site is prevented by a “closed” conformation of the activation loop (A-loop), a structurally conserved mobile segment containing key regulatory tyrosines that blocks substrate access to the catalytic niche but cannot be cis-phosphorylated because of steric hindrance. Upon ligand-induced stabilization of receptor homodimers, trans-phosphorylation of the A-loop by a neighboring protomer mediates destabilization of the closed A-loop conformation, thus removing autoinhibition and allowing for kinase activation (19, 20). In the case of Met, the A-loop contains two neighbor tyrosines, Tyr1234 and Tyr1235, that represent the major phosphorylation site of the receptor and are essential for the catalytic activity of the kinase. Substitution of either tyrosine with phenylalanine severely impairs wild-type kinase function (21, 22).

In this study, we investigate the molecular mechanisms underlying oncogenic conversion of Met by an immunological approach. To study the function of Tyr1234 and Tyr1235 in wild-type and mutant Met regulation, we developed antibodies specific for the differently phosphorylated forms of the Met A-loop. We provide evidence that three different transforming Met mutants, D1228H, D1228N, and M1250T, found in both

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† Due to space restriction required by the Journal, several publications in the field of tyrosine kinases relevant to this work could not be cited.

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1 The abbreviations used are: HGF, hepatocyte growth factor; RTK, receptor tyrosine kinase; A-loop, activation loop; p, phosphorylated.

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hereditary and sporadic human tumors are surprisingly not phosphorylated on Tyr1234.

By site-directed mutagenesis, we demonstrate that phosphorylation of Tyr1234 is dispensable for mutant Met biochemical and biological activity and that phosphorylation of Tyr1235 alone is sufficient to activate the mutant kinase. Consistent with this observation, the activation of mutant Met by auto-

Western Blot Analysis—Protein extracts were obtained by lysing cells in EB buffer (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol) in the presence of 1 mM sodium orthovanadate and a mixture of protease inhibitors (peptatin, leupeptin, aprotinin, soybean trypsin inhibitor, phenylmethylsulfonyl fluoride). Extracts were clarified by centrifugation, and protein concentration was determined using a BCA protein assay reagent kit (Pierce). Equal amounts of total protein extract (1 mg) were immunoprecipitated using anti-Met monoclonal antibodies (DQ-13) (27) adsorbed on Sepharose-protein A beads (Amersham Biosciences) using rabbit anti-mouse immunoglobulins (Fierce). Immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by Western blotting using the appropriate anti-A-loop antibodies, anti-phosphotyrosine antibodies (UBI, Lake Placid, NY), anti-Met polyclonal antibodies (C-12, Santa Cruz Biotechnology, Santa Cruz, CA), or anti-epidermal growth factor receptor antibodies (BD Transduction Laboratories, San Diego, CA). Bound antibodies were detected using the appropriate peroxidase-conjugated secondary antibodies and an ECL kit (both from Amersham Biosciences). Quantification of ECL signal was performed using a STORM apparatus with ImageQuant software (Amersham Biosciences).

Kinase Assays—For autokinase assays, the various Met proteins were produced by transient transfection in COS cells, extracted with EB buffer in the absence of orthovanadate, and then immunoprecipitated with anti-Met antibodies as described above. Immunoprecipitated proteins were washed in dephosphorylation buffer (100 mM Tris, pH 5.0, 10 mM MgCl2, 0.1 mM ZnCl2, 100 mM NaCl, 0.2 mM mg/ml bovine serum albumin, 0.1 mM sodium orthovanadate) and then dephosphorylated using purified calf alkaline phosphatase (2 units/50 μl, Promega, Madison, WI) for 1 h at 16°C. Dephosphorylated proteins were then washed five times with kinase buffer (25 mM HEPES, pH 7.1, 5 mM MgCl2, 100 mM NaCl). The phosphorylation reaction was performed in 50 μl of kinase buffer containing [γ-32P]ATP (5 Cpm/μl) and 10 μM unlabeled ATP for the indicated times at room temperature. The reaction was stopped by the addition of boiling Laemmli buffer. Samples were resolved by SDS-PAGE on a 8% polyacrylamide gel and then transferred to a Hybond nitrocellulose membrane (Amersham Biosciences). The radioactive filter was analyzed by autoradiography and quantified using a STORM apparatus with ImageQuant software. After autoradiography, the membrane was analyzed by Western blot using anti-Met polyclonal antibodies as described above and kinase activity was normalized on Met protein amount. For exogenous substrate kinase assays, the various Met proteins were prepared and dephosphorylated as described above. The kinase reaction was performed by incubating immunoprecipitated proteins with increasing concentrations of myelin basic protein (Sigma) in kinase buffer containing 10 μM unlabeled ATP and [γ-32P]ATP (5 Cpm/μl) for 15 min at 4°C. The reaction was blocked by the addition of reducing Laemmli buffer containing 10 mM EDTA. Samples were resolved by SDS-PAGE on a 8–12% gradient polyacrylamide gel. Following electrophoresis, the lower portion of the gel containing myelin basic protein was cut, dried, and analyzed by autoradiography. The upper portion of the gel containing Met protein was excised and then subjected to nondenaturing gel electrophoresis, analyzed by Western blot using anti-Met antibodies, and quantified as described above. Myelin basic protein phosphorylation was quantified using a STORM apparatus as described above, and values were normalized on Met protein levels. For preactivation experiments, immunoprecipitated Met proteins were incubated in kinase buffer containing 40 mM unlabeled ATP for 2 h at room temperature.

Sequential Phosphorylation of Tyr1235 and Tyr1234—To determine the phosphorylation kinetic Tyr1235 and Tyr1234, wild-type Met protein was prepared and dephosphorylated as described above. Dephosphorylated Met was subjected to an autophosphorylation reaction in kinase buffer containing 0.1 μM unlabeled ATP on ice and for the indicated times. The reaction was stopped by the addition of boiling Laemmli samples were resolved by SDS-PAGE on a 8% polyacrylamide gel, transferred to a Hybond nitrocellulose membrane, and then analyzed by Western blot using anti-AL-pTyr1235, anti-AL-pTyr1234, or anti-Met antibodies as described above.

RESULTS

Generation of Anti-phospho-A-loop Antibodies—To gain new insights into the mechanism of Met regulation, we generated antibodies against three different synthetic peptides corresponding to amino acids 1229–1242 of the Met A-loop. Each peptide had a distinct pattern of tyrosine phosphorylation: peptide-AL (not phosphorylated); peptide-AL-pTyr1234 (phosphorylated Tyr1234); and peptide-AL-pTyr1235 (phosphorylated Tyr1235).

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Tyr\textsuperscript{1235}. New Zealand White rabbits were immunized with the different peptides, and immunoglobulins from positive bleeds were purified by repeated affinity steps as described under "Experimental Procedures." The immunospecificity of purified antibodies was analyzed by Western blotting using COS cells transiently expressing wild-type Met as well as mutant forms of Met lacking Tyr\textsuperscript{1234}, Tyr\textsuperscript{1235}, or both. Because of very high levels of expression, exogenous RTKs produced in this system undergo spontaneous clusterization and trans-autophosphorylation. As shown in Fig. 1, left panel, anti-AL antibodies recognized the Met protein regardless of its phosphorylation status. In contrast, anti-AL-pTyr\textsuperscript{1234} and anti-AL-pTyr\textsuperscript{1235} antibodies were immunoreactive only with Met proteins containing a phosphorylated tyrosine in positions 1234 and 1235, respectively. In GTL16 gastric carcinoma cells that overexpress both Met and epidermal growth factor receptor (EGFr) antibodies. Right panel, A549 lung carcinoma cells express moderate Met levels and therefore retain HGF responsivity. The lower band (p145 Met) is the mature form of Met, whereas the upper band (p170 Met) corresponds to the immature Met precursor. In normal cells, only p140 Met is exposed at the membrane and becomes tyrosine-phosphorylated, but in overexpressing conditions (e.g. in COS cells), large amounts of p170 accumulates in the secretory pathway, thus resulting in increased tyrosine phosphorylation of the p170 form as well.

**Oncogenic Mutations Overcome the Requirement for Tyr\textsuperscript{1234} Phosphorylation**—To test this hypothesis, we mutagenized Tyr\textsuperscript{1234}, Tyr\textsuperscript{1235}, or both to phenylalanine in wild-type or mutant Met and analyzed kinase activity of the engineered receptors in both autophosphorylation assays (Fig. 3A) and kinase assays using an exogenous substrate (Fig. 3B). Because the D1228H and D1228N behaved similarly in all of the analyses performed, only the results relative to D1228H will be shown hereafter. As previously reported (21, 22), wild-type Met activity strongly relied on the presence of both tyrosines. In contrast, the kinase activity of D1228H Met and M1250T Met was not affected by the Y1234F substitution. Unexpectedly, also the Y1235F substitution did not reduce mutant kinase activity in the presence of Tyr\textsuperscript{1234}. However, this is easily explained by reciprocal compensating phosphorylation of the two neighbor tyrosines. In fact, Tyr\textsuperscript{1234} becomes phosphorylated in mutant Met when Tyr\textsuperscript{1235} is missing (Fig. 4). In any case, kinase activity of both D1228H Met and M1250T Met decreased dramatically upon mutagenesis of both Tyr\textsuperscript{1234} and Tyr\textsuperscript{1235} to phenylalanine, indicating that mutant Met requires at least one of the two tyrosines for function. This was also observed in biological assays aimed at measuring the transforming activity of the mutant Met proteins. NIH3T3 cells, which produce HGF (see Refs. 16 and 14), were transfected with plasmids encoding for wild-type or mutant Met, and the number of transformed foci was determined after 2 weeks. As shown in Table I, the transforming ability of D1228H Met and M1250T Met was not affected by a single tyrosine substitution but severely impaired by mutagenesis of both Tyr\textsuperscript{1234} and Tyr\textsuperscript{1235}. Therefore, onco-
genic mutations in the kinase domain of Met overcome the requirement of phosphorylation of one but not two key tyrosine residues in the A-loop.

**Oncogenic Mutations Lower the Threshold for Kinase Activation**—These results suggest that the D1228H and M1250T oncogenic amino acid substitutions achieve “partial” independence from activating signals mediated by A-loop tyrosine phosphorylation. This would predict that mutant forms of Met become activated more easily and earlier than wild-type Met. This was tested by an autophosphorylation time-course experiment in which receptors are forced to homodimerize by a specific antibody (21). Immunoprecipitated wild-type or mutant Met was accurately dephosphorylated to increase molecular homogeneity and then incubated with saturating concentrations of [32P]ATP for different times. The extent of autophosphorylation was determined by SDS-PAGE followed by autoradiography and quantified as described under “Experimental Procedures.” As Fig. 5A shows, M1250T Met and D1228H Met autophosphorylated at a substantially higher rate compared with wild-type Met. However, autophosphorylation eventually reached saturation, and in the long term (~2 h), all of the receptors were phosphorylated to the same extent. Because intrinsic kinase activity of mutant versus wild-type Met is normally measured using unphosphorylated receptors and an exogenous substrate (see Fig. 3B) (14, 15, 21), these data would be compatible with a scenario in which oncogenic forms of Met display higher kinase activity because they reach complete activation before wild-type Met. To test this hypothesis, a kinase assay was performed in parallel using (a) dephosphorylated Met or (b) preactivated Met (obtained by preincubating the immunoprecipitated receptors with ATP for 2 h). In accordance with the mechanism hypothesized, receptor preactivation using ATP completely abrogated the biochemical advantage of mutant Met over wild-type Met (Fig. 5B). All together, these data suggest that oncogenic mutations do not elicit constitutive activation of the Met kinase but rather decrease the threshold for kinase activation.

**Increased Autophosphorylation Occurs in the Presence of Stimulating Signals Only**—These results prompted us to test whether oncogenic mutations per se would result in increased receptor autophosphorylation in the context of a non-overexpressing cell system. Recombinant Met receptors were engineered to contain both an oncogenic mutation (D1228H or M1250T) and a deletion in the HGF-interacting domain in the extracellular portion of the molecule (16). NIH3T3 cells stably expressing the engineered receptors (ΔMet) or the correspond-
ing unmodified receptors (Met) were analyzed by Western blotting using anti-phospho-A-loop antibodies to determine Met activation. Fig. 6 shows the results of this analysis. Remarkably, the mutant forms of Met displayed increased A-loop phosphorylation compared with wild-type Met only when their ability to interact with endogenous HGF was preserved. All of the ΔMet receptors showed the same low levels of A-loop phosphorylation regardless of their genetic status. This cannot be due to the lack of membrane exposure or kinase inactivation in the ΔMet proteins, because these engineered receptors have been shown to be properly exposed at the cell surface, to respond to ligand-mimetic antibodies directed against the extracellular portion of the Met β-chain, and to be completely functional in kinase assays (16). Furthermore, ΔMet receptors containing the D1228H or M1250T mutation failed to transform NIH3T3 fibroblasts (Table I). Therefore, consistent with the data showed in Fig. 2, MLP29 panel, and the results obtained in kinase assays, oncogenic mutations result in increased auto-phosphorylation and acquisition of transforming ability only in the presence of activating signals.

**DISCUSSION**

In this study, we generated antibodies specific for the differently phosphorylated forms of the Met A-loop. Using these antibodies, we were able to show that activation of wild-type Met proceeds through the sequential phosphorylation of Tyr\(^{1235}\) and Tyr\(^{1234}\). The "independency" of mutant Met kinase from Tyr\(^{1234}\) phosphorylation does not explain the lack of Tyr\(^{1234}\) phosphorylation. This lack of phosphorylation probably reflects a different spatial orientation of the two A-loop tyrosines within the catalytic niche. Molecular modeling analysis of the Met A-loop revealed that the mean solvent-accessible surface of Tyr\(^{1235}\) is significantly higher than that of Tyr\(^{1234}\) (data not shown), suggesting that upon receptor dimerization, Tyr\(^{1235}\) is trans-phosphorylated before Tyr\(^{1234}\). This was tested in an auto-phosphorylation experiment performed in controlled conditions. De-phosphorylated wild-type Met was incubated with a limiting concentration of ATP to slow down the autocatalytic reaction. Autokinase activity was stopped at increasing time points, and the extent of Tyr\(^{1234}\)/Tyr\(^{1235}\) phosphorylation was determined by Western blotting using anti-AL-pTyr\(^{1234}\) and anti-AL-pTyr\(^{1235}\) antibodies. As shown in Fig. 7, the phosphorylation of Tyr\(^{1235}\) appeared first and then proceeded at approximately a 2-fold higher rate compared with Tyr\(^{1234}\) phosphorylation. This is consistent with a higher accessibility of Tyr\(^{1235}\) and confirms our hypothesis that, during kinase trans-activation, Tyr\(^{1235}\) is statistically phosphorylated before Tyr\(^{1234}\).
**Fig. 7. Sequential phosphorylation of Tyr1235 and Tyr1234.** Wild-type Met was incubated with a limiting concentration of ATP (0.1 mM) for increasing times, and the extent of Tyr1235 and Tyr1234 phosphorylation was determined by Western blotting using phospho-specific anti-A-loop antibodies.

From a biochemical viewpoint, the observation that Tyr1235 is phosphorylated before Tyr1234 helps to explain why mutant Met is not phosphorylated on Tyr1234. When two receptors meet at the cell surface, trans-phosphorylation may occur if their interaction is stable enough. The most exposed tyrosine (i.e. Tyr1235) has the higher chance to become a substrate of the adjacent receptor, but its phosphorylation is not sufficient to release autoinhibition in wild-type Met. Kinase activation is not achieved until a second phosphorylation event occurs on Tyr1234. Once both tyrosines have been phosphorylated, the A-loop loses its interactions with the kinase body, assuming an open conformation. At this point, the kinase domain undergoes a major conformational change and the catalytic niche becomes accessible to the next functional substrate of the activated kinase, conceivably the receptor tail containing the unique docking "super-site" (28).

This dual switch control system may have evolved to ensure a tight regulation of the Met kinase, a protein mastering vital biological processes in physiological conditions but leading to neoplasia when inappropriately activated. Oncogenic mutations subvert this control system not by directly activating the kinase but by overcoming the requirement for the second phosphorylation step. Therefore, in a mutant receptor, the phosphorylation of Tyr1235 is sufficient to remove A-loop-mediated autoinhibition and to elicit kinase activation. Once the enzyme is in the active state, the open conformation of the A-loop and the accessibility of the catalytic site to the many substrates of the Met signal transduction cascade may prevent phosphorylation of Tyr1234, which is no longer necessary in any case.

The notion that an oncogenic Met does not represent a constitutively active kinase but rather a deregulated enzyme that gets more easily activated is central to explain the unique biological features of mutant Met-driven tumorigenesis. In living cells, RTKs are subjected to opposite stimuli aimed at activating the kinase on one hand and at controlling its activity on the other hand by feedback mechanisms. In these conditions, an amino acid substitution lowering the threshold for activation will certainly result in a net shift toward the active form. However, as supported by our results, this will manifest only in the presence of positive stimuli and, in fact, mutant Met can display its oncogenic potential only upon stimulation with HGF (16).

In vivo, mutations in the met gene do not elicit a dramatic tumorigenic effect per se but clearly "prime" the affected individual or tissue to the development of cancer (10–13). Interestingly, individuals harboring a germ line met mutation develop mainly renal tumors despite the ubiquitous expression of the mutant gene (10). HGF is present in the human body mostly in the form of inactive precursor (pro-HGF) that is converted to an active factor upon local up-regulation of specific pro-HGF convertases (29). One of the most characterized pro-HGF convertases is urokinase (30), which is produced mainly if not solely by the kidney epithelium. All together, these observations suggest that active HGF is still an in vivo limiting factor for mutant Met-driven tumorigenesis and our data provide a molecular explanation for it.

The dual switch mechanism described in this work is similar in principle but asymmetric de facto to Knudson’s model for tumor suppressor gene inactivation (31). Indeed, in both cases two events have to accumulate before the aberrant phenotype is expressed. From a functional viewpoint, there is some homology between a “double loss of function” required for tumor suppressor gene inactivation and a “double removal of autoinhibitory signals” required for Met activation. In both systems, one event is provided genetically and the second is provided somatically, and this may explain some of the clinical features associated with mutant met that are unusual for a dominant oncogene. For example, long latency and incomplete penetrance usually reflect the requirement for loss of heterozygosity. In the case of met, it could be more appropriate to speak about “gain of homozygosity,” because duplication of the chromosome bearing the mutant met allele is invariably selected during tumor progression (17, 18). This leads to both higher expression of the mutant protein and increased autocrine stimulation because HGF and met lie both on chromosome 7 (this is a peculiarity not shared by other oncogenic RTKs).

This unique biologic scenario finds a molecular explanation in the results presented here, which depict mutant Met dormant in a semi-activated state, waiting for a “wake” signal to unleash its oncogenic and transforming potential.

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