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

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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 Tyr^{1235} and Tyr^{1234} in the activation loop and that mutagenesis of either tyrosine dramatically impairs kinase function. Surprisingly, oncogenic Met mutants never become phosphorylated on Tyr^{1234} despite their high enzymatic activity, and mutagenesis of Tyr^{1234} 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 therefore 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 embryo 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, cosegregate 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, Tyr^{1234} and Tyr^{1235}, 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 Tyr^{1234} and Tyr^{1235} 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.

hereditary and sporadic human tumors are surprisingly not phosphorylated on Tyr^{1234} despite their high kinase activity. By site-directed mutagenesis, we demonstrate that phosphorylation of Tyr^{1234} is dispensable for mutant Met biochemical and biological activity and that phosphorylation of Tyr^{1235} alone is sufficient to activate the mutant kinase. Consistent with this observation, the activation of mutant Met by autophosphorylation occurs faster compared with wild-type Met, and if the proteins get preactivated with ATP before the assay, the kinase activity of wild-type Met becomes comparable with that of mutant Met. Importantly, mutant Met displays increased tyrosine phosphorylation *in vivo* only if its ability to interact with HGF is preserved. These data suggest that oncogenic mutations in the *met* gene do not activate the kinase *per se* but simply lower the threshold for activation, explaining why a "second hit" is required by mutant Met to unleash its oncogenic potential.

EXPERIMENTAL PROCEDURES

Generation of Anti-phospho-A-loop Antibodies—Polyclonal antibodies were generated against the following synthetic peptides corresponding to amino acids 1229–1242 of human Met: peptide-AL, MYDKEYYSVHNKTG; peptide-AL-$pTyr^{1234}$, MYDKEpYYSVHNKTG; and peptide-AL-$pTyr^{1235}$, MYDKEYpYSVHNKTG. New Zealand White rabbits were immunized with peptides as described previously (23). Sera of immunized animals were purified by four consecutive steps of affinity chromatography as follows. In the first step, total immunoglobulins were purified using a Sepharose-protein A column (Amersham Biosciences) and eluted with 50 mM glycine, pH 2.7. Following neutralization with Tris-HCl, pH 8.0, and dialysis against phosphate-buffered saline, immunoglobulins (second step) were deprived of antibodies against non-phosphorylated A-loop by adsorption on a column of immobilized peptide-AL. The flow-through from step 2 was dialyzed against phosphate-buffered saline and then deprived of general anti-phosphotyrosine antibodies (step 3) using a column of phosphotyrosine-agarose (Sigma). In the last step, the flow-through from step 3 was applied to a column containing the phosphopeptide used for immunization and anti-phospho-A-loop-antibodies were purified by affinity. For purification of antibodies against non-phosphorylated A-loop, immunoglobulins from step 1 were applied directly onto a column of immobilized peptide-AL, purified by affinity, and then deprived of general anti-phosphotyrosine antibodies as described in step 3. Peptides were coupled to Affi-Gel 10 (Bio-Rad) at a density of 2.5 mg/ml according to the manufacturer's instructions. All of the antibodies were eluted from peptide columns with 50 mM glycine, pH 2.7, and immediately neutralized with Tris-HCl, pH 8.0. Purified antibodies were dialyzed against phosphate-buffered saline containing 10% glycerol and stored at −80 °C.

Site-directed Mutagenesis—The generation of the following Met mutants has been described: Y1234F Met, Y1235F Met, and Y1234F Y1235F Met (21); K1228A Met (24); D1228H Met, D1228N Met, and M1250T Met (15); and ΔMet (16). Starting from these mutants, all of the other combination mutants (D1228H Y1234F Met, D1228H Y1235F Met, D1228H Y1234F Y1235F Met, M1250T Y1234F Met, M1250T Y1235F Met, M1250T Y1234F Y1235F Met, D1228H ΔMet, and M1250T ΔMet) were generated using standard PCR and genetic-engineering techniques. Mutants were subcloned into the expression vector pMT2 (21) for transient expression or pCEV (16) for stable expression.

Cell Culture and Transfection—COS simian kidney epithelial cells (ATCC, Manassas, VA) and MLP29 mouse hepatocyte precursors (25) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Sigma). A549 lung carcinoma cells (ATCC) and GTL16 cells (26) were maintained in RPMI 1640 medium plus 10% fetal bovine serum. NIH3T3 mouse fibroblasts were grown in Dulbecco's modified Eagle's medium plus 10% calf serum (Colorado Serum Co., Denver, CO). Transient transfection of COS cells and stable transfection of MLP29 cells were performed using Lipofectin reagent (Invitrogen) according to the manufacturer's instructions. Focus formation assays were performed as described previously (16). Cells (1.5×10^5 cells/100-mm plate) were transfected with the appropriate pCEV plasmid (10 μg/plate) by the calcium-phosphate method in the presence of 10% calf serum. After 24 h, serum concentration was reduced to 5% and cells were cultured for an additional 14 days. Foci were scored following staining with Giemsa dye. HGF stimulation was performed on serum-starved MLP29 cells for 10 min at 37 °C using 100 ng/ml baculovirus-produced human recombinant HGF.

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 (pepstatin, 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 (Pierce). 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 8.0, 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 100 mM NaCl, 0.2 mg/ml bovine serum albumin) and dephosphorylated using purified shrimp 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 $MgCl_2$, 100 mM NaCl). The phosphorylation reaction was performed in 50 μl of kinase buffer containing [γ-^{32}P]ATP (5 Ci/sample) 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 mM unlabeled ATP and [γ-^{32}P]ATP (5 Ci/sample) 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 the Met protein was transferred to a nitrocellulose membrane, 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 Tyr^{1235} and Tyr^{1234}—To determine the phosphorylation kinetic Tyr^{1234} and Tyr^{1235}, 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 buffer. 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-$pTyr^{1234}$, anti-AL-$pTyr^{1235}$ antibodies, 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-$pTyr^{1234}$ (phosphorylated Tyr^{1234}); and peptide-AL-$pTyr^{1235}$ (phosphorylated

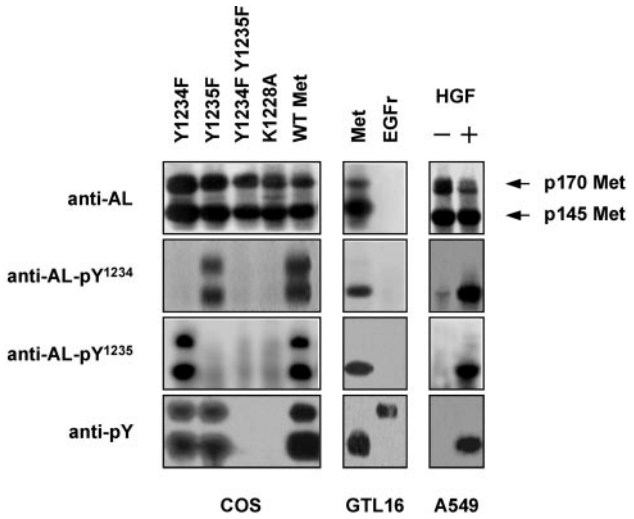

FIG. 1. **Generation of antibodies specific for distinct phosphorylated forms of the Met A-loop.** *Left panel*, COS cells transiently overexpressing the indicated Met proteins were analyzed by Western blot using anti-A-loop antibodies or anti-phosphotyrosine antibodies (*anti-pY*) following immunoprecipitation with anti-Met antibodies. K1228A Met is a kinase-inactive mutant. *WT Met*, wild-type Met. *Middle panel*, antibody specificity was tested in GTL16 gastric carcinoma cells following immunoprecipitation with anti-Met antibodies or anti-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.

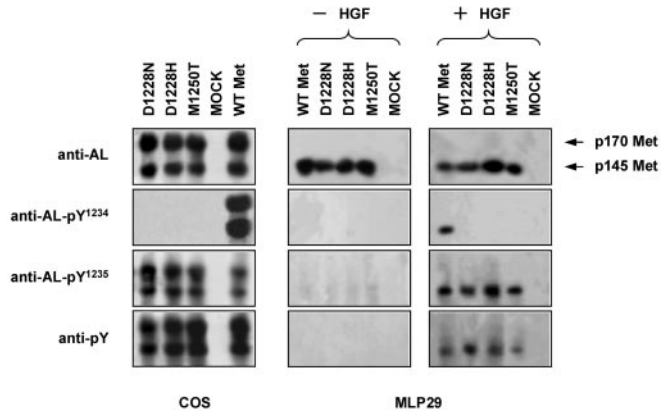

FIG. 2. **Oncogenic forms of Met are not phosphorylated on Tyr^{1234}.** *Left panel*, COS cells transiently overexpressing D1228H Met, D1228N Met, M1250T Met, or wild-type (*WT*) Met were analyzed by Western blotting as described in Fig. 1. *Right panel*, MLP29 epithelial cells stably expressing moderate levels of the indicated Met proteins were analyzed as above in the absence of growth factors or following stimulation with HGF for 10 min.

Tyr^{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^{1234}, Tyr^{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^{1234} and anti-AL-pTyr^{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, all of the antibodies were specific for Met (*central panel*). Immunoreactivity with Met was also observed in A549 lung adenocarcinoma cells that express moderate levels of receptor and therefore require ligand stimulation for Met autophosphorylation (*right panel*). It can be concluded that the generated antibodies can specifically discriminate among differently phosphorylated forms of the Met A-loop.

Oncogenic Forms of Met Are Not Phosphorylated on Tyr^{1234}—To shed light onto the molecular mechanisms underlying oncogenic conversion of Met, we analyzed the phosphorylation status of three representative mutant forms of Met commonly found in both hereditary and sporadic human tumors: D1228H Met, D1228N Met, and M1250T Met (11, 12). COS cells transiently expressing wild-type or mutant Met were analyzed by Western blotting as described above. Remarkably, this analysis revealed that all of the mutants analyzed are not phosphorylated on Tyr^{1234} (Fig. 2, *COS panel*). This is surprising because, as already stated, phosphorylation of both Tyr^{1234} and Tyr^{1235} is an absolute requirement for activation of the Met kinase (21, 22). Failure of the antibodies to recognize the mutant proteins cannot be the result of conformational changes of the kinase, because the immunoblotting procedure involves the boiling of samples in ionic detergent plus reducing agent and electrophoresis in denaturing conditions. Furthermore, the lack of Tyr^{1234} phosphorylation in mutant Met is not an artifact due to overexpression in COS cells, because epithelial cells (MLP29) stably expressing moderate levels of Met display the same pattern of immunoreactivity upon ligand stimulation (Fig. 2, *MLP29 panel*). Because the D1228H, D1228N, and M1250T Met mutants possess high catalytic and transforming activity (15–17), these data suggest that phosphorylation of Tyr^{1234} is dispensable for their function.

Oncogenic Mutations Overcome the Requirement for Tyr^{1234} Phosphorylation—To test this hypothesis, we mutagenized Tyr^{1234}, Tyr^{1235}, or both to phenylalanine in wild-type or mutant Met and analyzed kinase activity of the engineered receptors in both autophosphorylation assays (Fig. 3*A*) and kinase assays using an exogenous substrate (Fig. 3*B*). 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^{1234}. However, this is easily explained by reciprocal compensating phosphorylation of the two neighbor tyrosines. In fact, Tyr^{1234} becomes phosphorylated in mutant Met when Tyr^{1235} is missing (Fig. 4). In any case, kinase activity of both D1228H Met and M1250T Met decreased dramatically upon mutagenesis of both Tyr^{1234} and Tyr^{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^{1234} and Tyr^{1235}. Therefore, onco-

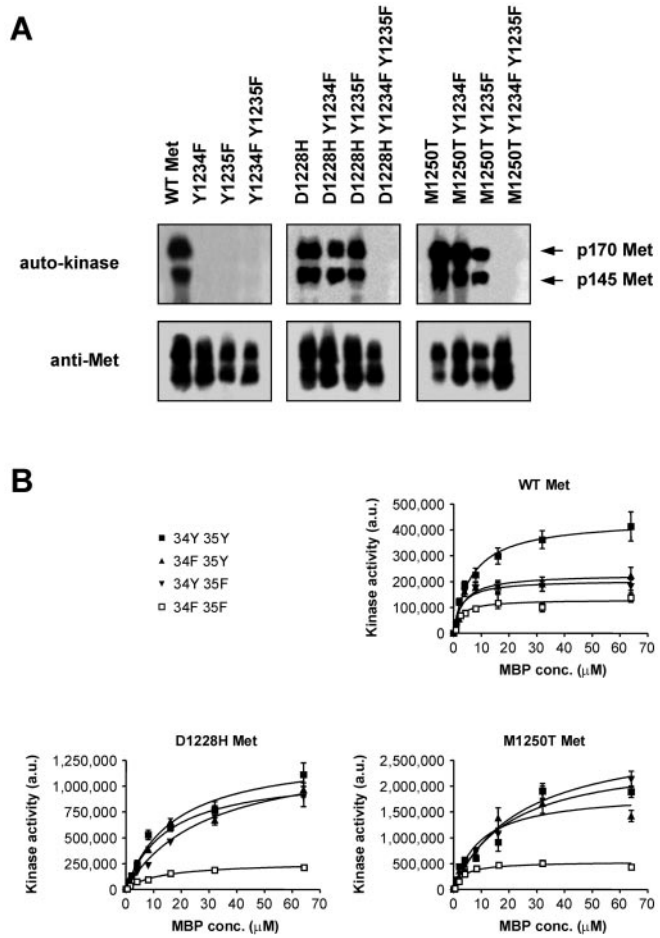

FIG. 3. **Oncogenic forms of Met require phosphorylation of only one A-loop tyrosine.** *A*, COS cells transiently expressing the indicated Met forms were analyzed for Met autokinase activity (*upper panel*). Met protein was normalized using anti-Met antibodies (*lower panel*). *B*, kinase activity of the various Met proteins using a saturating concentration of ATP and increasing concentrations of myelin basic protein (*MBP conc.*) as an exogenous substrate. Values on the *y* axes refer to the same arbitrary scale. *a.u.*, arbitrary units.

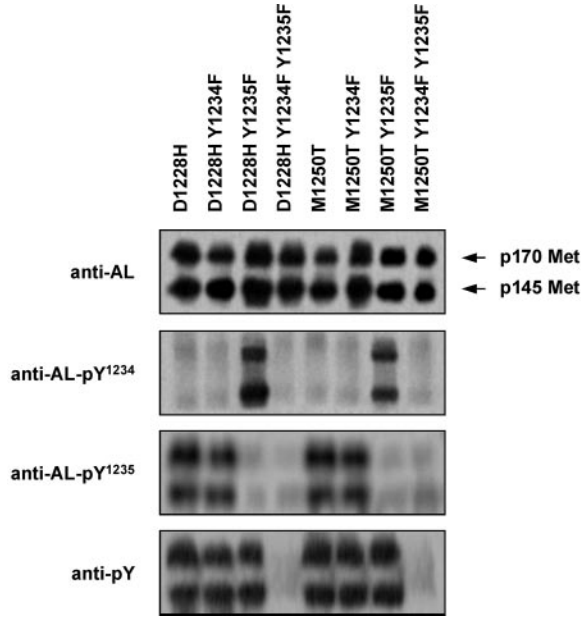

FIG. 4. **Tyr^{1234} becomes phosphorylated in mutant Met when Tyr^{1235} is missing.** COS cells transiently overexpressing the indicated Met forms were analyzed by Western blot using anti-A-loop antibodies or anti-phosphotyrosine antibodies (*anti-pY*) as described in Fig. 1.

TABLE I
Focus formation assay

NIH3T3 cells were transfected as described previously (16) with plasmid constructs encoding for the indicated Met proteins, and the number of transformed foci was scored after two weeks (mean ± S.D., $n = 3$). WT, wild type.

Plasmid construct	Number of foci
Empty vector	3.7 ± 1.2
WT Met	4.7 ± 1.2
D1228H	46.7 ± 5.5
D1228H Y1234F	40.3 ± 4.2
D1228H Y1235F	39.0 ± 6.0
D1228H Y1234F Y1235F	10.0 ± 2.8
M1250T	73.3 ± 2.5
M1250T Y1234F	52.7 ± 4.7
M1250T Y1235F	51.7 ± 8.7
M1250T Y1234F Y1235F	10.3 ± 3.1
ΔMet	3.3 ± 1.5
ΔMet D1228H	4.7 ± 2.1
ΔMet M1250T	4.3 ± 2.1

genic mutations in the kinase domain of Met overcome the requirement of phosphorylation of one but not two key tyrosine residue 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" independency 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 [^{32}P]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. 5*A* 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. 3*B*) (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. 5*B*). 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-

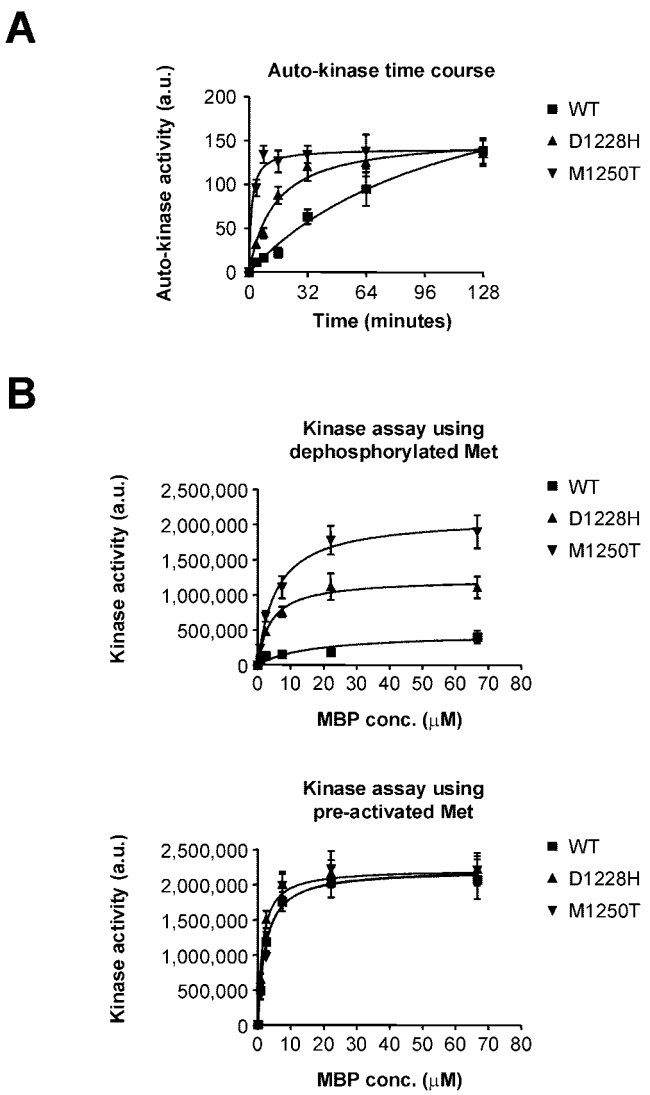

FIG. 5. **Oncogenic mutations do not constitutively activate the Met kinase but lower the threshold for activation.** *A*, immunoprecipitated wild-type or mutant Met was incubated in the presence of [^{32}P]ATP for increasing times, and the extent of autophosphorylation was determined by autoradiography. *a.u.*, arbitrary units. *B*, the ability of wild-type or mutant Met to phosphorylate an exogenous substrate (*MBP conc.*) was measured either using dephosphorylated proteins or following preactivation with ATP.

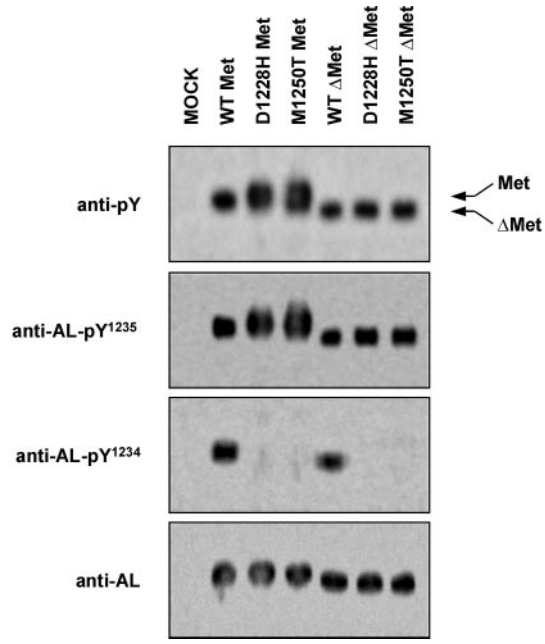

FIG. 6. **Increased autophosphorylation occurs in the presence of activating signals only.** NIH3T3 cells that produce HGF (see Refs. 14 and 16) stably expressing wild-type (*WT*) or mutant Met in its integral form (Met) or containing a deletion in the HGF-interacting domain (ΔMet) were analyzed by Western blotting as indicated following immunoprecipitation with anti-Met antibodies.

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 autophosphorylation and acquisition of transforming ability only in the presence of activating signals.

Sequential Phosphorylation of Tyr^{1235} and Tyr^{1234}—Yet still 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 autophosphorylation experiment performed in controlled conditions. Dephosphorylated 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}.

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} and that oncogenic mutant forms of Met found in human cancer fail to be phosphorylated on Tyr^{1234} despite their high kinase activity. Biochemical and biological assays aimed at measuring the activity of wild-type and mutant Met revealed that oncogenic amino acid substitutions in the Met kinase overcome the requirement for phosphorylation of one of the two A-loop tyrosines, thus suggesting that a mutant receptor has a lower threshold for activation compared with wild-type Met.

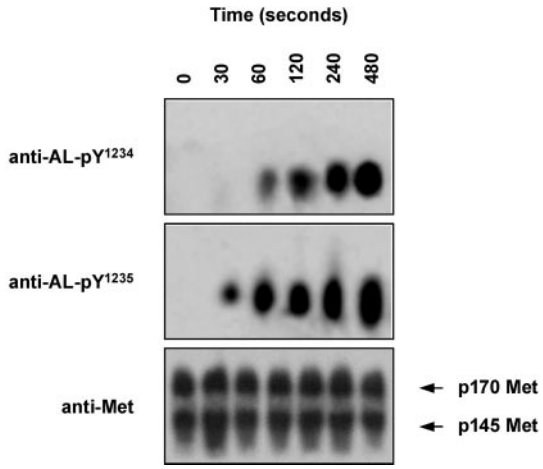

FIG. 7. **Sequential phosphorylation of Tyr^{1235} and Tyr^{1234}.** Wild-type Met was incubated with a limiting concentration of ATP (0.1 μM) for increasing times, and the extent of Tyr^{1234} and Tyr^{1235} phosphorylation was determined by Western blotting using phospho-specific anti-A-loop antibodies.

From a biochemical viewpoint, the observation that Tyr^{1235} is phosphorylated before Tyr^{1234} helps to explain why mutant Met is not phosphorylated on Tyr^{1234}. When two receptors meet at the cell surface, *trans*-phosphorylation may occur if their interaction is stable enough. The most exposed tyrosine (*i.e.* Tyr^{1235}) 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 Tyr^{1234}. Once both tyrosines have been phosphorylated, the A-loop loosens 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 Tyr^{1235} 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 Tyr^{1234}, 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 symmetric *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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REFERENCES

1. Rubin, J. S., Bottaro, D. P., and Aaronson, S. A. (1993) *Biochim. Biophys. Acta* **1155,** 357–371
2. Trusolino, L., and Comoglio, P. M. (2002) *Nature Rev. Cancer* **4,** 289–300
3. Bladt, F., Riethmacher D., Isenmann S., Aguzzi A., and Birchmeier, C. (1995) *Nature* **376,** 768–771
4. Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E., and Birchmeier, C. (1995) *Nature* **373,** 699–702
5. Matsumoto, K., and Nakamura, T. (1997) *CIBA Found. Symp.* **212,** 198–211
6. Miyazawa, K., Shimomura, T., Naka, D., and Kitamura, N. (1994) *J. Biol. Chem.* **269,** 8966–8970
7. Trusolino, L., and Comoglio, P. M. (2002) *J. Clin. Invest.* **109,** 857–862
8. Vande Woude, G. F., Jeffers, M., Cortner, J., Alvord, G., Tsarfaty, I., Resau, J. (1997) *CIBA Found. Symp.* **212,** 119–130
9. Birchmeier, W., Brinkmann, V., Niemann, C., Meiners, S., DiCesare, S., Naundorf, H., and Sachs, M. (1997) *CIBA Found. Symp.* **212,** 230–240
10. Schmidt, L., Duh, F. M., Chen, F. *et al.* (1997) *Nat. Genet.* **16,** 68–73
11. Schmidt, L., Junker, K., Nakaigawa, N. *et al.* (1999) *Oncogene* **18,** 2343–2350
12. Park, W. S., Dong, S. M., Kim, S. Y. *et al.* (1999) *Cancer Res.* **59,** 307–310
13. Di Renzo, M. F., Olivero, M., Martone, T., Maffé, A., Maggiora, P., Stefani, A. D., Valente, G., Giordano, S., Cortesina, G., and Comoglio, P. M. (2000) *Oncogene* **19,** 1547–1555
14. Jeffers, M., Schmidt, L., Nakaigawa, N., Webb, C. P., Weirich, G., Kishida, T., Zbar, B., and Vande Woude, G. F. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 11445–11450
15. Bardelli, A., Longati, P., Gramaglia, D., Basilico, C., Tamagnone, L., Giordano, S., Ballinari, D., Michieli, P., and Comoglio, P. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 14379–14383
16. Michieli, P., Basilico, C., Pennacchietti, S., Maffé, A., Tamagnone, L., Giordano, S., Bardelli, A., and Comoglio, P. M. (1999) *Oncogene* **18,** 5221–5231
17. Zhuang, Z., Park, W. S., Pack, S., Schmidt, L., Vortmeyer, A. O., Pak, E., Pham, T., Weil, R. J., Candidus, S., Lubensky, I. A., Linehan, W. M., Zbar, B., and Weirich, G. (1998) *Nat. Genet.* **19,** 66–69
18. Fischer, J., Palmedo, G., von Knobloch, R., Bugert, P., Prayer-Galetti, T., Pagano, F., and Kovacs, G. (1998) *Oncogene* **17,** 733–739

19. Hubbard, S. R., Mohammadi, M., and Schlessinger, J. (1998) *J. Biol. Chem.* **273,** 11987–11990
20. Weiss, A., and Schlessinger, J. (1998) *Cell* **94,** 277–280
21. Longati, P., Bardelli, A., Ponzetto, C., Naldini, L., and Comoglio, P. M. (1994) *Oncogene* **9,** 49–57
22. Rodrigues, G. A., and Park, M. (1994) *Oncogene* **9,** 2019–2027
23. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Crepaldi, T., Prat, M., Giordano, S., Medico, E., and Comoglio, P. M. (1994) *J. Biol. Chem.* **269,** 1750–1755
25. Medico, E., Mongiovì, A. M., Huff, J., Jelinek, M. A., Follenzi, A., Gaudino, G., Parsons, J. T., and Comoglio, P. M. (1996) *Mol. Biol. Cell* **7,** 495–504
26. Giordano, S., Di Renzo, M. F., Ferracini, R., Chiado-Piat, L., Comoglio, P. M. (1988) *Mol. Cell. Biol.* **8,** 3510–3517
27. Ruco, L. P., Ranalli, T., Marzullo, A., Bianco, P., Prat, M., Comoglio, P. M., and Baroni, C. D. (1996) *J. Pathol.* **180,** 266–270
28. Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G., and Comoglio, P. M. (1994) *Cell* **77,** 261–271
29. Gak, E., Taylor, W. G., Chan, A. M., and Rubin, J. S. (1992) *FEBS Lett.* **311,** 17–21
30. Naldini, L., Tamagnone, L., Vigna, E., Sachs, M., Hartmann, G., Birchmeier, W., Daikuhara, Y., Tsubouchi, H., Blasi, F., and Comoglio, P. M. (1992) *EMBO J.* **11,** 4825–4833
31. Knudson, A. G. (1986) *Annu. Rev. Genet.* **20,** 231–251