Oligomerization-induced Modulation of TPR-MET Tyrosine Kinase Activity

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Phosphorylation, although necessary, may not be sufficient to fully activate many receptor tyrosine kinases (RTKs). Oligomerization-induced conformational changes may be necessary to modulate the kinetic properties of RTKs and render them fully functional. To investigate this regulatory mechanism, recombinant TPR-MET, a functionally active oncoprotein derivative of the RTK c-MET, has been expressed and purified for quantitative enzymatic analysis. This naturally occurring oncoprotein contains the cytoplasmic domain of c-MET fused to a coiled coil motif from the nuclear pore complex (TPR). cytoMET, the monomeric analog of TPR-MET, has also been expressed and purified for comparative enzymatic analysis. ATP and peptide substrates have been kinetically characterized for both TPR-MET and cyto-MET. Significantly, phosphorylated TPR-MET has smaller $K_m$ values for ATP ($K_m^{ATP}$) and peptide substrates ($K_m^{peptide}$) and a larger $K_{cat}$ relative to phosphorylated cytoMET. This provides the first direct evidence that receptor oligomerization and not simply activation loop phosphorylation modulates RTK enzymatic activity. The ATP dissociation constants ($K_d^{ATP}$) for the two enzymes also displayed significant differences. In contrast, the $K_f$ values for the ATP competitive inhibitor staurosporin are similar for the two phosphorylated enzymes. These results suggest that much of the oligomerization-induced kinetic changes occur with respect to peptide substrate binding or catalytic efficiency. The possibility that oligomerization-induced conformational changes occur within the cytoplasmic domain of receptor tyrosine kinases has significant implications for structure-based design of RTK inhibitors and the development of a detailed mechanistic model of RTK activation.

Stimulation of receptor tyrosine kinases (RTKs) by ligand binding initiates an intracellular signaling cascade that can induce cellular differentiation, proliferation, and/or migration, among other cellular responses (1). Binding of a cognate extracellular ligand to a monomeric RTK induces receptor oligomerization and, in turn, autophosphorylation of tyrosine residues within the cytoplasmic tail of the receptor (1–3). The phosphorylated form of the receptor recruits signaling molecules through Src homology 2 or phosphotyrosine binding domains, and subsequent activation of these molecules initiates intracellular signaling cascades (1, 4).

The molecular details of how RTK oligomerization regulates its function are incompletely understood. It is known that receptor oligomerization enhances autophosphorylation of tyrosine residues within the kinase regulatory domain and that this phosphorylation results in localized conformational changes that increase the kinase activity of the receptor (5–7). However, regulatory domain phosphorylation, although necessary, may not be sufficient to fully activate the kinase (8, 9). Additional oligomerization-induced conformational changes may be necessary to increase the kinase catalytic activity and render the RTK fully functional. Evidence for this level of regulation is provided by studies with phosphatase inhibitors such as peroxovanadate compounds. Posner et al. (7) demonstrated that unstimulated insulin receptors possess basal kinase activity but are unable to fully mimic ligand-stimulated insulin receptor autophosphorylation and signaling in cultured hepatoma cells or hepatic microsomes following peroxovanadate treatment (10). Similarly, pervanadate treatment of the platelet-derived growth factor (PDGF) receptor is able to induce autophosphorylation in the absence of PDGF but is unable to increase the activity of the receptor toward exogenous substrate without the presence of PDGF (9). Studies with polyclonal antibodies have qualitatively shown that a phosphorylated monomeric receptor does not possess the ability to phosphorylate exogenous substrate in the same manner as the oligomeric receptor (8). All of these studies would indicate that there is some unique functional characteristic contained within the oligomeric RTK that is not present in the monomeric receptor.

Biochemical characterization of RTKs has been performed using both recombinant truncated kinase domains and cytoplasmic domains (5, 11–14). Cheng and Koland (14) showed that the cytoplasmic domain of the EGF receptor has an almost 10-fold greater $K_f$ for an ATP analog than a carboxyl terminus deletion mutant of the cytoplasmic tail (14), demonstrating that the isolated kinase domain may not be a sufficient model for receptor function. Murray et al. (5) were able to determine kinetic parameters for phosphorylated and unphosphorylated Tie2 cytoplasmic kinase domain and showed that phosphorylation resulted in a 2–5-fold decrease in substrate $K_m$. Parast et al. (12) also showed an order of magnitude increase in the catalytic activity of the phosphorylated VEGFR2 tyrosine kinase domain versus the non-phosphorylated receptor. These
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The TPR-MET oncoprotein provides a unique opportunity to address this question. TPR-MET is a naturally occurring oncoprotein resulting from a fusion between TPR (a coiled-coil domain derived from the nuclear pore complex) and the cytoplasmic domain of c-MET (6, 15, 16) (Fig. 1). c-MET, the RTK for hepatocyte growth factor/scatter factor, has been shown to be involved in angiogenesis, placental and liver development, B-cell differentiation, and embryogenesis, and altered c-MET function has been implicated in multiple neoplastic disorders (17). Targeted overexpression of c-MET has also been shown to cause hepatocellular carcinoma in transgenic mice (18). The TPR-MET oncoprotein is oligomerized through the TPR domain, which results in a constitutively active tyrosine kinase (6) that has the ability to produce mammary hyperplasia and carcinoma, as well as multiple other neoplasms in transgenic mice (18). The isolated cytoplasmic domain of c-MET (cytoMET) has kinase activity; however, this protein cannot transform NIH3T3 cells and is, therefore, functionally inactive (6). These studies suggest there may be kinetic differences between functionally active TPR-MET and functionally inactive cytoMET. Here we present a detailed analysis that conclusively shows phosphorylated TPR-MET and phosphorylated cytoMET have significantly different kinetic properties. Because these two proteins differ in their oligomerization state, this implies that oligomerization-induced conformational changes modulate the activity of RTKs, and these changes are necessary to fully activate the receptor.

EXPERIMENTAL PROCEDURES

Materials and Reagents—All restriction endonucleases were from New England BioLabs, Taq pfu was from Promega, the Bac-to-Bac baculovirus expression system, SF-900II serum-free medium, fetal bovine serum, and 100 X antibiotic/antimycotic were purchased from Invitrogen. ATP, NaVO₄, DTT, HEPES, and staurosporin were purchased from Roche Applied Science. SAM² biotin capture membrane and SigmaTECT (Sigma-Aldrich) protein tyrosine kinase assay system, substrate 1, biotin-EEEEAYGWLD were purchased from Promega. Complete protease inhibitor mixture was purchased from Roche Applied Science. SAM² biotin capture membrane and SigmaTECT (Sigma-Aldrich) protein tyrosine kinase assay system, substrate 1, biotin-EEEEAYGWLD were purchased from Promega. Complete protease inhibitor mixture was purchased from Roche Applied Science. PMPr41 (biotinylated phosphotyrosine analog) was synthesized and purified in the University of Texas Medical Branch (UTMB) Protein Chemistry Core Facility, and its identity was verified by mass spectrometry. MANT-AMPPnP (a fluorescent non-hydrolyzable derivative of ATP) was synthesized and purified by the UTMB Organic Chemistry Core Laboratory, and its identity was verified by mass spectrometry. Anti-human Met antibody was purchased from Santa Cruz Biotechnology, Inc. (sc-161), and anti-phosphotyrosine (clone 4G10) and the anti-phosphorylated MET activation loop antibodies were from Upstate Biotechnology, Inc.

Construction of Recombinant Baculovirus Vectors—The tpr-met oncogene was a generous gift from Dr. Morag Park (McGill University). Both tpr-met and cytoMET were amplified using PCR of the tpr-met containing pXM vector. A carboxyl-terminal hexahistidine tag and BamHI and EcoRI restriction sites were added during PCR. The PCR product was gel-purified, digested, and ligated into the pFastBac shuttle plasmid. The sequence was verified in the shuttle plasmid using automated sequencing (UTMB Protein Chemistry Core Laboratory). Clones with verified sequences were transformed into DH10Bac cells; transposition into baculovirus DNA was monitored by loss of β-galactosidase activity. Recombinant baculovirus DNA was purified by isopropanol precipitation and successive ethanol washes and used for transfection of confluent Sf9 cells. The recombinant baculovirus obtained from the Bac-to-Bac cloning protocol was amplified to a titer of >1 × 10⁸ pfu/ml before infections for protein expression. Amplifications were carried out using 5 × 10⁶ cells/ml and a multiplicity of infection of 0.05 for 96 h at 27 °C. Titer was verified using serial dilution plaque assays in duplicate.

Protein Expression and Purification—Both TPR-MET and cytoMET proteins were expressed and purified using similar protocols. Sf9 insect cells were propagated at 27 °C in spinner flasks using SF-900II serum-free medium supplemented with 5% fetal bovine serum and 0.5 × antibiotic/antimycotic; cell density was maintained between 5 × 10⁵ and 3 × 10⁶ cells/ml for propagation. Protein expression was initiated by infecting Sf9 cells (2 × 10⁵ cells/ml) with recombinant baculovirus at a multiplicity of infection of 5. Cells were pelleted 72 h post-infection and stored at –78 °C. Frozen cell pellets were thawed on ice, resuspended with chilled TBSC (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% CHAPS) supplemented with 1 mM DTT and 1 × complete protease inhibitor mixture, and lysed by sonication. The lysate was cleared by centrifugation 30,000 × g for 30 min at 4 °C. The cleared lysate was incubated overnight at 4 °C with pre-equilibrated Ni-NTA beads (washed with TBSC). After binding, the beads were washed successively with TBSC + 250 mM NaCl and TBSC + 500 mM NaCl for 5 min imidazole. Protein was eluted with TBSC + 500 mM imidazole. Purification was monitored by SDS-PAGE with the resulting bands visualized with Coomassie Blue staining. Protein concentrations were determined by absorbance at A₂₈₀ with molar extinction coefficients calculated from the aromatic residue content of the proteins (19).

To ensure complete autophosphorylation of the recombinant kinases, the eluent was dialyzed at 4 °C against PBSC (50 mM sodium phosphate, pH 6.5, 150 mM NaCl, 0.5% CHAPS, 1 mM DTT) supplemented with 50 μM ATP, 25 mM MgCl₂ and 5 mM MnCl₂. A second dialysis was performed against PBSC and 5 mM EDTA. After dialysis, the proteins were further purified over gel filtration column (Phenomenex BIO-SEP SEC-3000 column) in PBSC + 5 mM EDTA or an anion exchange (POROS 10 HQ) column with salt as the eluent. Both proteins were characterized with gel filtration chromatography (Phenomenex BIO-SEP SEC-3000 column) to determine their oligomerization state.

Fluorescence Studies of TPR-MET and cytoMET—Binding of MANT-AMPPnP to fluorescently tagged, non-hydrolyzable derivatives of TPR-MET and cytoMET was measured using a Fluorolog-3 spectrofluorometer, model PL3.22–23. Quenching of the fluorescence from the five common tryptophan residues found in both TPR-MET and cytoMET was measured for increasing concentrations of MANT-AMPPnP. All data were measured at 10 °C with an excitation wavelength (λₓ) of 280 nm. Emission data were collected from 300 to 357 nm for both the sample and a reference cuvette with buffer and ligand. Fluorescence was corrected for inner filter effects by measuring the absorbance of the protein/ligand solutions at the excitation and emission wavelengths. The quenching of tryptophan fluorescence occurring upon the addition
of MANT-AMP-PnP was assumed to be proportional to the amount of protein-bound MANT-AMP-PnP present. The data were fitted using the following equation to obtain dissociation constants: \\
\[ \Delta F = \Delta F^0 - 1 - (Q[MANT-AMP-PnP] + [MANT-AMP-PnP]) \]
Where \( \Delta F \) represents the fluorescence of the sample minus the background cuvette, \( F^0 \) is the initial protein fluorescence in the absence of ligand, \( Q \) is the maximum quenching of the protein during the experiment, and \( K_d \) is the dissociation constant. The data were plotted as \( \Delta F/F^0 \text{ versus } [\text{MANT-AMP-PnP}] \) and fit using non-linear least squares regression analysis to the above equation with the GraphPad Prism program and \( K_d \) and \( Q \) as fitting parameters. To determine the dissociation constant for ATP (\( K_{d,ATP} \)), titrations of enzyme with MANT-AMP-PnP were performed in the presence of a fixed amount of ATP. The \( K_{d,ATP} \) can then be determined by solving the equation: \( K_d \) apparent = \( K_{d,ATP} \) (1 – (ATP/\( K_{d,ATP} \))). To determine total active protein, the quenching of total fluorescence was assumed to be proportional to the amount of MANT-AMP-PnP bound to the protein, and the dissociation constant was determined in independent experiments. The total protein (\( I[E] \)) can then be found by solving the quadratic equation: \\
\[ (1 - \Delta F/\Delta F^0) = (\frac{I[E]}{[\text{MANT-AMP-PnP}] + K_d}) - (\frac{I[E]}{[\text{MANT-AMP-PnP}] + K_d})^2 - 4(\frac{I[E][\text{MANT-AMP-PnP}])^{1/2}/4(I[E]). \]

**Kinetic Assays**—All kinetic assays were performed at room temperature unless otherwise noted. Ni-NTA-purified TPR-MET (or cytoMET) was pre-incubated for 10 min at 4 °C in kinase reaction buffer (50 mM Hepes, pH 7.3, 100 mM NaCl, 25 mM MgCl₂, 5 mM MnCl₂, 5 mM β-glycerophosphate) with 1 μM ATP and 10 μM freshly prepared DTT added. Kinase reactions were carried out in kinase reaction buffer supplemented with 0.1 mM NaN₃, 1 μM γ-[32P]ATP and 1 mM DTT, with ATP and tyrosine-containing substrate added to desired concentrations. Reactions were initiated by addition of enzyme (final concentration 1–10 μM) to a complete reaction mixture. At time points during the steady state linear range of the reaction, aliquots were removed from the reaction mix, quenched with 5 μM guanidine hydrochloride, and applied to streptavidin-coated polyvinylidene difluoride membranes (SAM biotin capture membrane; Promega). The membranes were sequentially washed with 2 mM NaCl, 2 mM NaCl + 1% H₃PO₄, distilled water, and 70% EtOH. Membranes were dried, and incorporated radiolabel was measured using Cerenkov counting on a Hekellett Packard LS300 liquid scintillation counter. Non-weighted least squares linear regression lines were calculated for the linear range of each of individual reaction, and the program GraphPad Prism was used to calculate steady state rates. Each time point was measured in duplicate, and each linear range contained from four to eight points. Kinetic parameters were calculated from non-linear least squares fitting to a Michaelis-Menten equation using the program GraphPad Prism.

**Inhibitor Studies**—To determine the \( K_d \) of the known ATP competitive inhibitor staurosporin, linear ranges were calculated (as described above) in the presence of a known amount of inhibitor. Staurosporin was initially dissolved to a final concentration of 1 mM in Me₂SO and then subsequently diluted into kinase reaction buffer at the desired final concentration. Steady state rates were compiled to generate rate curves, and unweighted non-linear least squares regression analysis was used to determine \( K_d \) of inhibitor. Determining this value at multiple inhibitor concentrations allowed calculation of \( K_d \) by comparing to the previously calculated \( K_d \) for ATP using the formula: \\
\[ K_d = \frac{[I][K_d]}{[I][K_d] + 1) \] (22).

**RESULTS**

**Purification of TPR-MET and cytoMET**—The expression of carboxyl-terminal hexahistidine-tagged TPR-MET and cytoMET in SF9 cells was accomplished by infection with recombinant baculovirus as described under “Experimental Procedures.” Both proteins were isolated from the soluble fraction of the cell lysate and were purified to ≥95% homogeneity as evaluated by Coomassie Blue staining (Fig. 2). Fig. 2B shows a Western blot analysis of both TPR-MET and cytoMET after purification. Both proteins positively react against a commercially available anti-human c-MET antibody that was derived from a peptide corresponding to the carboxyl-terminal tail of the c-MET receptor. Both TPR-MET and cytoMET are phosphorylated as demonstrated from their recognition by anti-phosphotyrosine antibody 4G10 and an antibody specific for the phosphorylated activation loop of c-MET. Furthermore, incubation of this phosphorylated enzyme with 4μg[32P]ATP revealed no further incorporation of phosphate into either TPR-MET or cytoMET. This result is expected, because both proteins were incubated from 4 h to overnight at 4 °C with ATP and MgCl₂ during the purification process (see “Experimental Procedures”).

Enzymatic characterization showed that kinetic variables for each enzyme recovered after elution from either the Ni-NTA or gel filtration columns were similar (data not shown). Both proteins were subjected to analysis on gel filtration columns to determine their oligomerization state. TPR-MET (molecular mass, ~62 kDa) eluted at a volume corresponding to an observed molecular mass between 100 and 250 kDa; no protein was apparent in the void volume or in fractions corresponding to monomeric molecular mass. This size range would correspond to an oligomer of a dimer, trimer, or tetramer for the TPR-MET protein. Different concentrations of TPR-MET were used in the kinase assays, and each time identical \( K_{d,ATP} \) and \( K_{m,peptide} \) were obtained. Thus, even if multiple oligomerization states were present their kinetic properties were identical. cytoMET (molecular mass, ~43 kDa) eluted at a volume corresponding to a molecular mass of ~40 kDa, consistent with a monomeric cytoMET species.

**Binding Studies**—Binding studies of TPR-MET and cytoMET for MANT-AMP-PnP allowed determination of substrate affinities and subsequent determination of the binding active enzyme fraction in the purified protein samples. The latter measurement is necessary to accurately determine the \( k_{cat} \) value for TPR-MET and cytoMET. The quenching of intrinsic tryptophan fluorescence by MANT-AMP-PnP was measured for both phosphorylated TPR-MET and cytoMET. Four of the five tryptophan residues are within the carboxyl-terminal kinase domain of the both TPR-MET and cytoMET, as determined by
sequence analysis and comparison with the known structure of the insulin receptor (20). These residues were accessible to quenching by the MANT nucleotide whose absorption maximum at 355 nm overlaps with the tryptophan emission maximum in both TPR-MET and cytoMET at 335 nm. A fixed amount of protein was titrated with increasing amounts of MANT-AMPPnP, and the quenching was followed from 300 to 387 nm, to avoid interference with MANT emission (Fig. 3A). The area under the curve was integrated, and, after inner filter effects were accounted for, normalized fluorescence was plotted against [MANT-AMPPnP], and the data were fit with non-linear least squares regression analysis (Fig. 3, B and C). Results from the binding studies are listed in Table I. The binding constants for MANT-AMPPnP ($K_{d,MANT}$) are 9.1 $\mu$M for TPR-MET and 8.7 $\mu$M for cytoMET, which are statistically identical for the two enzymes. To determine ATP binding constants ($K_{d,ATP}$), the titrations were repeated with 10 $\mu$M ATP added. The apparent $K_d$ with ATP present, when coupled to the known $K_{d,MANT}$ can yield the $K_{d,ATP}$ as described under “Experimental Procedures.” This analysis reveals a slight decrease in $K_{d,ATP}$ when comparing the oligomeric TPR-MET to the monomeric cytoMET indicating an oligomerization-dependent change in the conformation of the ATP binding site, independent of phosphorylation of the activation loop.

**Kinetic Activity**—To ensure complete autophosphorylation of the enzymes and to separate the autophosphorylation reaction from the exogenous substrate kinase reactions, both TPR-MET and cytoMET were separately incubated with ATP, MgCl$_2$, and MnCl$_2$ prior to use in kinetic studies. The steady state kinetic properties of the fully phosphorylated form of TPR-MET and cytoMET with respect to ATP and exogenous tyrosine substrates were examined. TPR-MET and cytoMET were both recognized by phosphotyrosine-specific antibodies before the kinase reaction. However, neither enzyme underwent additional autophosphorylation during the course of these reactions, as demonstrated by the inability of either enzyme to incorporate phosphate from radiolabeled [$\gamma^{32}$P]ATP (data not shown). It was also necessary to maintain adequate reducing conditions (DTT concentration $> 1$ mM) to ensure full activity of the kinases. Thus, both enzymes were reduced and fully phosphorylated before determining their kinetic properties with exogenous substrates.

The steady state linear range for formation of phosphorylated exogenous substrate occurred between 2 and 50 min for both phosphorylated TPR-MET and cytoMET. This range is similar to the linear range reported for ligand-stimulated immunoprecipitated c-MET (11). Linear ranges were analyzed using an unweighted linear least squares regression analysis within the GraphPad Prism program (see Fig. 4A and 5A). For each reaction, between four and eight separate time points were examined in duplicate to determine the reaction rate; all regression lines fit the data with $r^2$ values greater than 0.9. Fig. 4A and Fig. 5A show representative kinetic data with fixed enzyme and ATP concentrations and increasing SignaTECT 1 peptide concentrations. The slopes of the calculated regression lines provided steady state velocities from which to construct Michaelis-Menten rate curves (see Fig. 4B and Fig. 5B) for SignaTECT 1. An identical method was used to construct rate curves for the tyrosine-containing peptide substrate, PepTyr-489, and ATP (see Fig. 4, C and D and Fig. 5, C and D). Each data point in the Michaelis-Menten rate curves represents the mean of two to five independent experiments. Michaelis-Menten equations were fit to the experimental data using unweighted non-linear least squares regression, and kinetic parameters were derived from the curve for each kinase reaction.

Table II summarizes the $K_m$ values for ATP ($K_{m,ATP}$). When measuring the influence ATP has on the reaction rate for
phosphorylation of exogenous substrates, the ATP concentration was varied from 2 to 800 μM whereas the peptide substrate (SignaTECT 1 or PepTyr-489) concentration was held constant at 400 μM. Both TPR-MET and cytoMET followed Michaelis-Menten kinetics with respect to ATP at constant peptide substrate concentrations. The $K_{m, \text{ATP}}$ value for TPR-MET was determined to be 35.7 μM, which is in agreement with the reported value for immunoprecipitated c-MET (11). The $K_{m, \text{ATP}}$ value for cytoMET was 70.4 μM, a factor of two increase compared with TPR-MET and a difference that was statistically significant. The value of $K_{m, \text{ATP}}$ was independent of peptide substrate used in the kinase reaction. The TPR-MET and cytoMET $K_{m, \text{ATP}}$ values are well below reported intracellular concentrations of ATP; thus, ATP concentration would likely not be a significant regulatory mechanism for activation of the enzyme.

The $K_{m}$ values for peptide substrates ($K_{m, \text{peptide}}$) are summarized in Table II. Several peptide substrates were tested for their activity with TPR-MET and cytoMET. PepTyr-489, a biotinylated peptide (residues Val-483–Pro-497 of TPR-MET) that includes the in vivo phosphorylation site Tyr-489 in the carboxyl-terminal domain of TPR-MET, and SignaTECT 1, a biotinylated peptide containing a single tyrosine residue, were found to be the best substrates out of a number of different tested peptides for the in vitro kinase reactions. Both TPR-MET and cytoMET followed Michaelis-Menten kinetics when varying peptide substrate concentrations from 1 to 1000 μM and holding ATP concentration constant at 250 μM. The $K_{m, \text{peptide}}$ values of PepTyr-489 and SignaTECT 1 with TPR-MET are 11.8 and 40.7 μM, respectively. In contrast, the $K_{m, \text{peptide}}$ values of PepTyr-489 and SignaTECT 1 with cytoMET are of 67.3 and 231 μM, respectively. The $K_{m, \text{peptide}}$ values, whether the peptide was derived from the cytoplasmic tail of the receptor (PepTyr-489) or a nonspecific peptide containing one tyrosine residue, both increased by almost 6-fold for cytoMET compared with TPR-MET (Table I).

The $V_{max}$, $k_{cat}$, and $k_{cat}/K_{m}$ values are reported for both enzymes in Table II. The active fraction of protein was defined as that portion able to bind ATP as determined by fluorescence measurements (see Table I and Fig. 3). Typically, 10–30% of the purified protein was able to bind nucleotide; the exact fraction that bound nucleotide was considered active and was determined just prior to kinetic analysis. Taking the active fraction determined for a particular sample and the calculated $V_{max}$ for a defined amount of enzyme allowed accurate calculation the kinase $k_{cat}$. The measured $k_{cat}$ for TPR-MET was 1.66 min$^{-1}$ and for cytoMET was 0.569 min$^{-1}$, showing an oligomerization dependant 3-fold increase in the rate of the reaction. The specificity constant ($k_{cat}/K_{m}$) for the oligomeric TPR-MET increased by greater than 5-fold for ATP and greater than 15-fold for peptide substrates when compared with cytoMET (Table II). These data would suggest there is a significant difference between the functional activity of oligomeric TPR-MET and monomeric cytoMET proteins, implying an oligomerization-induced conformational change affects the inherent kinetic activity of receptor tyrosine kinases.

**Inhibitor Studies**—To further understand active site differences between TPR-MET and cytoMET, the kinetics of a kinase reaction were performed in the presence of staurosporin. Staurosporin has been characterized as an ATP competitive inhibitor for multiple kinases (21), and the crystal structure for staurosporin within the ATP binding site of tyrosine kinases
Src (Protein Data Bank number 1BYG) and human lymphocyte-specific kinase (Protein Data Bank number 1QPJ) has been determined. Kinase reactions were performed as above, except that staurosporin was added to the reaction mixture to obtain final staurosporin concentrations within an order of magnitude of the expected $K_I$. Reactions were performed with 0, 0.5, and 1 nM staurosporin, 400 μM SignaTECT 1, and ATP concentrations varying from 10 to 800 μM. $K_m$, $ATP$ values ranged from 100 to 250 μM for TPR-MET in the presence of inhibitor (see Fig. 6A and Table III). $K_m$, $ATP$ values ranged from 200 to 500 μM for cytoMET in the presence of inhibitor (see Fig. 6B and Table III). $K_f$ values were then calculated according to Segel (22), with $K_f$ values of 0.19 and 0.15 nM obtained for TPR-MET and cytoMET, respectively (Table III). This small difference in $K_f$ was not statistically significant, as judged by detailed error analysis of the data (Table III).

### DISCUSSION

Numerous groups have demonstrated the important role of RTKs in both normal and diseased tissues (1–3). The mechanism and pathways RTKs use to transduce their signals has been exhaustively researched, but there is still little known about the actual molecular mechanism of how RTKs become activated and what is necessary and sufficient for receptor activation and signaling. To study the effect of oligomerization on receptor tyrosine kinase activity, we have used a functionally active fusion protein derivative of the cytoplasmic domain of the c-MET receptor, TPR-MET, and compared it to a functionally inactive protein, cytoMET, containing the cytoplasmic domain of the c-MET receptor (6).

The catalytic activities ($k_{cat}$) for oligomeric TPR-MET and monomeric cytoMET have been accurately determined. Signif-
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![Graph A](image1)

![Graph B](image2)

**Fig. 6.** Staurosporin inhibition of TPR-MET and cytoMET. Rate curves were generated as described under “Experimental Procedures” using varying ATP concentrations and constant peptide concentrations (250 μM SigmaTECT1). The inhibitors were mixed at the given concentrations into the reaction mixture before addition of enzyme. Panel A, inhibition of TPR-MET with staurosporin. Panel B, inhibition of cytoMET.

**TABLE III**

| Inhibition kinetics of TPR-MET and CytoMET |
|-------------------------------------------|
| TPR-MET and cytoMET were analyzed as described under “Experimental Procedures” to generate Michaelis-Menten curves in the presence of varying concentrations of staurosporin (Fig. 5). The $K_{\text{app}}$ values were calculated using nonlinear least squares regression analysis of the data from Fig. 5 in the presence of a known concentration of inhibitor. The $K_i$ values were then calculated using the following equation: $K_i = \frac{[I]}{[I]/(K_{\text{app}}/K_m) - 1}$ (22). Two-tailed $p$ values were calculated using a Student’s t distribution. |
| **$K_{\text{app}}$ (μM)** | **$K_i$ (μM)** | **$p$ Value** |
|-------------------------|----------------|--------------|
| **Staurosporin (nm)**   | **TPR-MET**    | **CytoMET**  | **p Value** |
| 0.0                     | 32.7 (4.78)    | 70.4 (10.9)  |              |
| 0.5                     | 102 (16.7)     | 244 (54.6)   |              |
| 1.0                     | 237 (41.5)     | 558 (41.8)   |              |
| $K_i$                   | 0.19 (0.043)   | 0.15 (0.033) | 0.41         |

significantly, functionally active TPR-MET has a 3-fold increase in $k_{\text{cat}}$ relative to functionally inactive cytoMET. In addition there was an $\sim$17-fold increase in the specificity constant ($k_{\text{cat}}/K_m$) for peptide and a greater than $\sim$6-fold increase in the specificity constant for ATP when comparing the relative activities of TPR-MET to cytoMET. The observed increase in specificity constant for TPR-MET relative to cytoMET was independent of peptide substrate used. This suggests that receptor oligomerization may modify the conformation of the catalytic site, thus leading to increased activity of the oligomeric receptor.

To further explore the relationship between oligomerization-induced changes in binding affinity and kinetic activity, we determined the $K_{i,\text{ATP}}$, $K_{m,\text{ATP}}$, and $K_{m,\text{peptide}}$ values for oligomeric TPR-MET and monomeric cytoMET. Interestingly, the binding of MANT-AMPPNP was identical for both enzymes, and the binding affinity of ATP ($K_{i,\text{ATP}}$) was $\sim$3-fold lower for TPR-MET when compared with cytoMET. In addition, $K_{m,\text{ATP}}$ was $\sim$2-fold lower for TPR-MET relative to cytoMET. These data suggest that the ATP binding site was similar for both TPR-MET and cytoMET, and thus, receptor oligomerization may only slightly modify the conformation of the receptor ATP binding site. In contrast, $K_{m,\text{peptide}}$ was $\sim$6-fold lower for TPR-MET relative to cytoMET, suggesting that oligomerization may induce conformational changes in the tyrosine substrate binding region. This would explain the large differences in $K_{m,\text{peptide}}$ values and catalytic activity observed between TPR-MET and cytoMET.

RTKs oligomerize in response to ligand binding, and this process facilitates autophosphorylation of the receptor on specific tyrosine residues (3). The question remains, does receptor oligomerization modulate the phosphorylation of downstream signaling molecules? Many groups have studied isolated kinase domains or intracellular regions of RTKs, and their work, as well as work presented here, demonstrates that isolated kinase domains are sufficient for enzymatic activity in vitro (5, 12, 13) (Fig. 5). Rodrigues and Park (6) demonstrated that the TPR-MET fusion oncprotein was kinase-active in vitro and functionally active in cell culture. In contrast, the monomeric cytoMET was kinase-active in vitro, but it was functionally inactive in cultured cells. Other studies (9, 23) have shown that monomeric RTKs can undergo autophosphorylation in cell culture in the presence of phosphatase inhibitors. These studies suggest that the monomeric form of the RTKs possess some basal kinase activity but are unable to fully mimic the effects of the oligomeric receptors. One possible explanation for this observation might be that oligomerization creates a local increase in concentration of tyrosine substrate for the kinase. In this hypothesis, it is not necessary for the oligomer to have any different kinetic properties from the monomer, as the increase in concentration of substrates would presumably increase the activity of the kinase to a sufficient level to induce downstream signaling. An alternative hypothesis is that oligomerization causes some structural alterations in the kinase domain that decreases the $K_m$ and increases the catalytic activity of the receptor such that downstream signaling is initiated. In this hypothesis, a defined interaction between the oligomeric subunits causes the conformational change in the receptor and the subsequent increase in its enzymatic activity. One possible structural explanation for this has been proffered by Hubbard et al. (2), where they suggest formation of a dimer may stabilize the kinase activation loop in a catalysis-favorable conformation. Our data support this latter hypothesis, as the oligomeric TPR-MET possesses increased catalytic activity, as measured by a decrease in $K_m$ and an increase in $k_{\text{cat}}$ compared with monomeric cytoMET. These two hypotheses are not mutually exclusive, as oligomerization likely does induce a local increase in tyrosine substrate concentration that may then be acted upon by a kinase with enhanced catalytic activity. It is also important to note that these experiments do not address the important point of dephosphorylation of the active kinase. Oligomerization may confer protection from dephosphorylation, as has been reported for the PDGF receptor (24), and this mechanism may play a role in downstream signaling through oligomeric, as opposed to monomeric, receptors.

$K_{m,\text{ATP}}$ and $K_{m,\text{peptide}}$ values have been measured for several kinases. Naldiini et al. (11) reported $K_{m,\text{ATP}}$ values of 36 μM for ATP and 1.5 μM for tyrosine-containing peptide substrates for both autophosphorylated immunoprecipitated e-MET and the im-
munoprecipitated cytoplasmic portion of the receptor. These reported $K_m$ values for both ATP-MET and immunoprecipitated c-MET. Importantly, we do not account for the observed differences in $K_m$ between TPR-MET and immunoprecipitated c-MET. Importantly, we see significant differences in $K_m$ between TPR-MET and monomeric cytoMET, whereas the immunoprecipitated receptors have identical $K_m$ values. It is likely that immunoprecipitation of the receptor modifies its conformation, either through artificial receptor oligomerization or some other mechanism, and thus immunoprecipitated receptors do not provide an accurate model of the monomeric receptor.

Other groups (8–10) have similarly reported that there is a difference in the ability of monomeric receptors to fully mimic oligomeric receptor activation in vitro and in cell culture. Posner et al. (7) demonstrated that pre-activation of the EGF receptor with epidermal growth factor led to a decrease in the apparent dissociation constant for both ATP and peptide substrates compared with non-activated receptors. Together, these data suggest that oligomerization, in addition to activation loop phosphorylation and protection from phosphatases (24), modulates the catalytic activity of receptor tyrosine kinases. It is likely that oligomerization induces conformational changes to the catalytic transition state, the ATP binding pocket, and/or the tyrosine-containing substrate binding site and therefore is important in determining the catalytic properties of the enzyme.

The activities of both enzymes were examined in the presence of the ATP competitive inhibitor staurosporin. A number of compounds that interact with the kinase ATP binding site have been discovered and kinetically characterized (21, 25). There is a wealth of kinetic (26) and crystallographic data (27, 28) demonstrating the role of staurosporin and related compounds as ATP competitive inhibitors in protein tyrosine kinases. In this work, we have shown that staurosporin acts as an ATP competitive inhibitor for both TPR-MET and cytoMET with statistically indistinguishable $K_i$ values for both enzymes (Table III). Thus, staurosporin binding more closely approximates the binding of MANT-ATP to the two kinases as opposed to the binding of ATP to these kinases, suggesting that staurosporin binding may be influenced by factors and contacts outside the ATP-binding interface.

Our data suggest that the increased $K_m$ values for the monomeric receptor relative to the oligomeric receptor results from a decrease in its catalytic efficiency. However, this does not strictly exclude the possibility that the monomeric receptor may also have a decreased affinity for peptide substrates relative to the oligomeric receptor. Previous work on EGF receptor (7, 11) demonstrated that the EGF-stimulated receptor acted with the same kinetic mechanism and efficiency as the non-stimulated receptor, but the affinities for various substrates

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