A transforming mutation enhances the activity of the c-Kit soluble tyrosine kinase domain

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An activating mutation (DY814) located in the catalytic domain of the c-Kit receptor has been found in mastocytomas from human, mouse and rat. We evaluated the enzymic properties of purified wild-type (WT) and DY814 tyrosine kinase domains expressed in *Pichia pastoris*. A linker encoding the Flag epitope was fused to c-Kit cDNA species, enabling affinity purification of the proteins with anti-Flag antibodies. Yeast lysates expressing DY814 contained multiple tyrosine-phosphorylated proteins, whereas WT lysates had no detectable tyrosine phosphorylation. Purification of the WT and mutant kinases in the presence of vanadate demonstrated that both enzymes undergo auto-phosphorylation. Kinetic analyses of WT and DY814 kinases indicated that at 20 nM enzyme concentration the mutation increases the specific activity 10-fold and decreases the apparent

K_m for ATP 9-fold. WT activity displayed a hyperbolic dependence on enzyme concentration, consistent with a requirement for dimerization or aggregation for activity. This activity was also enhanced by anti-Flag antibodies. In contrast, the dependence of DY814 activity on enzyme concentration was primarily linear and only marginally enhanced by anti-Flag antibodies. Gel-filtration analysis showed that the WT kinase migrated as a monomer, whereas the DY814 mutant migrated as a dimer. These results indicate that this point mutation promotes dimerization of the c-Kit kinase, potentially contributing to its transforming potential in mast cells.

Key words: dimerization, growth factor receptor, mastocytoma, *Pichia pastoris*.

INTRODUCTION

The c-Kit proto-oncogene encodes a receptor tyrosine kinase that is a member of the platelet-derived growth factor receptor family of receptors [1]. This family includes the colony-stimulating factor 1 receptor and the fms-like tyrosine kinase receptor and is characterized by an extracellular domain consisting of five immunoglobulin-like repeats, a single transmembrane domain and an intracellular kinase split by a hydrophilic sequence. The ligand for c-Kit has been cloned [2] and designated steel factor, mast cell growth factor, Kit ligand or stem cell factor. c-Kit is the gene product of the *W* locus (white spotting) in mice [3]. *W* mutations affect the intrinsic ability of haemopoietic stem cells, mast cells and other cells to undergo mitosis and proliferate. In contrast, mutations at the *Sf* locus encoding steel factor affect the microenvironment in which haemopoiesis occurs [4].

Dimerization or oligomerization of receptor tyrosine kinases is an immediate consequence of ligand binding. One result of this stable interaction between two or more receptors is auto-phosphorylation, probably due to transphosphorylation within the dimer [5]. The phosphorylated receptor then recruits and activates SH2-containing substrates that transmit signals downstream. Experiments with the platelet-derived growth factor receptor [6], epidermal growth factor receptor [5] and insulin receptor [7] showed that receptors dimerized by ligand, or aggregated by antibodies or lectins, have increased kinase activity as compared with non-dimerized or non-aggregated receptors. Although this enhancement in activity is well documented, the enzymic consequences of dimerization and autophosphorylation are not fully understood.

Several mutations resulting in the ligand-independent activation of receptors have been described. The primary effect of many of these mutations seems to be the promotion of

dimerization. For example, transforming mutations found in the RET receptor tyrosine kinase cause ligand-independent homodimerization resulting in multiple endocrine neoplasia type 2A [8]. Constitutive activation by mutations that cause receptor dimerization in the absence of ligand have been described for c-Neu [9], fibroblast growth factor receptor 2 [10], colony-stimulating factor 1 receptor [11] and erythropoietin receptor [12]. In addition, Rodrigues and Park [13] have demonstrated that the transforming tpr-met fusion protein is dependent on leucine zipper-mediated dimerization for its oncogenic activity.

A number of transforming mutations in the c-Kit gene have been observed in human and rodent tumours. A Val-560 → Gly mutation, located in the juxtamembrane region of the receptor, was identified in the c-Kit gene of the HMC-1 human mast cell leukaemia [14]. When a similar mutation was introduced into the murine c-Kit gene (Val-559) and expressed in Ba/F3 murine factor-dependent cells, this mutant stimulated factor-independent growth of the cells. Chemical cross-linking experiments with these cells demonstrated c-Kit dimers in the absence of ligand, indicating that the effect of this mutation is to promote ligand-independent dimerization [15]. Tsujimura et al. [16] described another activating mutation in the same region of the c-Kit gene found in the murine FMA3 mastocytoma. This mutation results in a deletion of seven amino acid residues (573–579). Chemical cross-linking analysis also demonstrated c-Kit dimers in the absence of ligand, again consistent with ligand-independent dimerization. More recently, a series of mutations have been observed in c-Kit genes found in human gastrointestinal tumours [17]. Of six mutations analysed, five were found to have either point mutations or deletions in the same region of the receptor. Analysis of these five mutated receptors also revealed ligand-independent activity, although the dimerization status of these receptors was not determined.

Abbreviations used: AOX1, alcohol oxidase 1; DTT, dithiothreitol; MBP, myelin basic protein; WT, wild-type.

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A point mutation in the catalytic-domain region of c-Kit has been identified in mouse [18], rat [19] and human [14,20] mast cell tumours. Mutations of murine c-Kit Asp-814 to either a Val or a Tyr residue, when expressed in factor-dependent cells, also cause factor-independent growth and tumorigenicity. In contrast with the results for the juxtamembrane mutations, chemical cross-linking studies were unable to demonstrate that the DV814 or DY814 mutation induces dimerization in the absence of ligand [15].

To examine the consequences of this mutation in c-Kit activation, we have expressed, purified and analysed the enzymic properties of wild-type (WT) and DY814 murine c-Kit cytoplasmic domains. Intracellular expression of the kinase domain was performed in the yeast *Pichia pastoris*. Our observations indicate that the DY814 mutation enhances the activity of the c-Kit catalytic domain in a manner that is consistent with the promotion of stable dimers. These results contribute to our understanding of the transforming mechanism of this mutation and further highlight the importance of dimerization in receptor tyrosine kinase activation.

EXPERIMENTAL

Materials

The *P. pastoris* expression system and vectors were purchased from InVitrogen. Murine c-Kit cDNA species encoding the WT receptor and the DY814 mutant were obtained from Dr. Alan Bernstein (Toronto, Ontario, Canada). A cDNA encoding the W⁴² mutant was kindly provided by Dr. Peter Besmer (New York, U.S.A.). Zymolyase-20T was purchased from ICN Bio-medicals. Culture media and reagents were purchased from Difco and Sigma. Oligonucleotides were purchased from Gibco. M2 anti-Flag antibodies and M2 anti-Flag gel were purchased from InterSciences. Affinity-purified alkaline phosphatase-conjugated goat anti-(mouse Ig) and anti-(rabbit IgG) were purchased from Jackson Laboratories; 4G10 monoclonal anti-phosphotyrosine antibodies were from UBI. Standards for gel electrophoresis and Bradford assays were purchased from Bio-Rad. Nitrocellulose membranes and p81 phosphocellulose were purchased from Whatman International Ltd.; filter plates were purchased from Canberra Packard. PMSF was purchased from Boehringer Mannheim; [γ -³²P]ATP and [γ -³³P]ATP were purchased from Dupont. Sephacryl S-200 was purchased from Pharmacia. Myelin basic protein (MBP), ATP and FPLC standards were purchased from Sigma.

Expression vectors

The *Pichia* integrating vector pHIL-D2 [21] was used to express the mouse intracellular domain of the c-Kit receptor. This vector contains the alcohol oxidase 1 (AOX1) promoter and gene, the histidinol dehydrogenase selectable marker, ampicillin resistance and an f1 origin of replication. The insertion of DNA C-terminal to the AOX1 promoter displaces the AOX1 gene and permits the identification of integrants by screening for a lack of growth on methanol medium owing to a loss of alcohol oxidase activity. The cDNA for murine c-Kit was subcloned into an intermediate plasmid and modified to contain an *NcoI* and an *EcoRI* restriction site at the N-terminus and C-terminus of the intracellular domain respectively. To create the *NcoI* site, an additional glycine residue was inserted between residues 551 and 552 of the sequence. Two complementary oligonucleotides encoding an initiating methionine, the Flag epitope [22], a tri-glycine linker and seven residues C-terminal to the juxtamembrane domain were synthesized: 5'-AAT TCA TGG ACT ACA AAG ACG ATG

ACG ATA AAG CAG GTG GCG GTT ACA AAT ACC TGC AGA AAC CC-3' and 5'-CAT GGG TTT CTG CAG GTA TTT GTA ACC GCC ACC TGC TTT ATC GTC ATC GTC TTT GTA GTC CA-3'. The linker was also flanked by *EcoRI* and *NcoI* restriction sites at the 5' and 3' ends respectively. The c-Kit cDNA and linker were ligated into pHIL-D2 as follows: the unique *EcoRI* site located C-terminal to the AOX1 promoter in pHIL-D2 was digested and ligated with the 2.1 kb *NcoI*-*EcoRI* fragment encoding the cytoplasmic domain of c-Kit in a timed reaction (12 min). The ligation product was gel-purified with GeneClean and then ligated with the linker overnight to complete the vector. Electroporation-competent DH5 α cells were transformed with the ligation mix and colonies were screened by mini-plasmid preparations and restriction digestion. Sequencing confirmed that pHIL-D2 contained one linker and one insert encoding the c-Kit cytoplasmic domain in the proper orientation and reading frame. A point mutation was found in the tri-glycine portion of the linker but this mutation did not affect the amino acid coding of the gene. The new yeast plasmid with linker and insert was named pLL701. Vectors containing the W⁴² and DY814 mutations were generated by exchanging fragments containing these mutations into pLL701. Both were confirmed by sequencing. The W⁴² mutation changes nt 2396 from an A to a G, resulting in an amino acid change from Asp to Asn. The DY814 mutation is a consequence of changing nt 2468 from a G to a T.

Generation of *P. pastoris* transformants

Stable transformants were generated by the homologous recombination of linear vector DNA into the host genome by using a two-step procedure. Initially, transformants were selected for histidinol dehydrogenase on histidine-deficient medium. Homologous recombinants were then identified by screening transformants for sensitivity to methanol. Colonies with high growth on glucose-based medium (MD medium) but minimal growth on methanol-based medium (MM medium) have integrated the linear DNA at the correct locus and no longer contain the AOX1 gene (his⁺, mut⁻ phenotype).

Spheroplasts were prepared from cultures of GS115 yeast cells in accordance with the procedure recommended by the manufacturer. Spheroplasts were transformed with *NorI*-linearized pLL701 and plated on histidine-deficient dextrose-based regeneration medium for 4–6 days at 30 °C. The viability of spheroplasts was also checked and confirmed to be optimal. Screening for AOX1-disrupted transformants was performed by sterile transfer of transformants to both MM and MD media followed by growth for an additional 2 days under the same temperature conditions. After 2 days, plates were scored for colonies that grew well on MD medium but not on MM medium.

Expression of c-Kit cytoplasmic domains in *Pichia pastoris*

Cultures were grown overnight in 100 ml of MGY medium (in which glycerol is provided as the carbon source). Cells were harvested by centrifugation and resuspended in an equivalent volume of MM medium to induce intracellular expression of the kinase. Induction was performed for 6 days and additional methanol was added to replenish the medium on days 2, 4, 5 and 6 of the induction. Aliquots (5 ml) of cells were removed on days 3–6 of the induction for lysate preparation. The D_{600} of each aliquot was determined and cells were washed in cold breaking buffer [50 mM sodium phosphate/1 mM PMSF/1 mM EDTA/5% (v/v) glycerol]. After being washed, cells were resuspended in a final volume at a D_{600} of 50. An equal volume of acid-washed glass beads was added and the solution was mixed by repeated

vortex-mixing for 30 s, alternating with 30 s on ice, for a total of 8 min. The sample was then centrifuged at 4 °C for 10 min to pellet the beads and cell membranes. The clear supernatant was transferred to a fresh tube and analysed by SDS/PAGE and Western blotting for expression levels of the protein. Clone 18, a his⁺/mut⁺ *Pichia* strain provided by Invitrogen, was used as a negative control for expression.

Purification of cytoplasmic domains by using M2 anti-Flag gel

Cultures (1 litre) were grown, induced and lysed as described above. Lysates were dialysed overnight in dialysis buffer [10 mM Hepes (pH 8.3)/150 mM NaCl/1 mM EDTA/10% (v/v) glycerol/1 mM dithiothreitol (DTT)]. Vanadate, when added, was at a concentration of 0.2 mM. Before purification, the lysate was redialysed in the same buffer minus the DTT for 2 h at 4 °C to prevent the reduction of antibody on the affinity purification column. Triton X-100 (2%, v/v) was added after dialysis and the lysate was centrifuged for 45 min at 250 000 *g* and 4 °C. After centrifugation, the supernatant was filtered through a 0.2 µm filter and incubated with rotation for 1 h at room temperature with 200 µl of M2 anti-Flag beads. The beads had been prewashed with 0.1 M glycine, pH 3, and then with dialysis buffer lacking DTT. The supernatant and beads were transferred to a column (0.8 cm × 4 cm) and the flow-through was collected. The column was washed with 10–20 column vol. of dialysis buffer lacking DTT. Bound protein was eluted with aliquots of 0.1 M glycine, pH 3, and immediately neutralized with Tris/HCl, pH 8. Fractions were pooled and redialysed overnight in dialysis buffer at 4 °C. Purified protein was divided into aliquots and stored at –80 °C.

Kinase assays *in vitro*

Reactions were performed for 10 min at 30 °C in a final volume of 50 µl in dialysis buffer with the following additional components as required: 10 mM MnCl₂, 100 µM MBP, [γ -³²P]ATP or [γ -³³P]ATP (10 µCi). Reactions were terminated by the addition of EDTA to 10 mM. A 10 µl sample of each reaction was dotted on p81 phosphocellulose paper immobilized in a Flexifilter plate (Packard). After all reactions had been dotted on, the filter was washed extensively in 75 mM phosphoric acid to remove any unincorporated ATP. The filter was left to dry and then replaced in the Flexifilter plate. Scintillation cocktail was added and radioactivity was measured in a TopCount scintillation counter (Packard).

SDS/PAGE analyses of kinase reactions *in vitro* were performed as follows: 5 × SDS gel loading buffer was added to the reaction mixture and the sample was boiled for 5 min before being loaded on an SDS/PAGE gel. After separation, the gel was incubated in 1 M KOH at 55 °C with shaking, to hydrolyse phosphoserine and phosphothreonine. After treatment with KOH, the gel was fixed by shaking at room temperature in 50% (v/v) methanol/10% (v/v) acetic acid for 30 min. Fixing was then repeated before drying at 80 °C for 2 h. The gel contents were detected by autoradiography with film or by PhosphorImager analysis.

Gel chromatography

Purified kinase domains in 10 mM Hepes (pH 8.3)/150 mM NaCl/1 mM EDTA/10% (v/v) glycerol/1 mM DTT/0.05% (v/v) Triton X-100 (buffer A) were applied to a Sephacryl S-200 column equilibrated with the same buffer attached to an FPLC apparatus (Bio-Rad) and eluted with the same buffer. The column was operated at 4 °C, at a flow rate of 0.25 ml/min;

0.5 ml fractions were collected. The void volume of the column was 10 ml. The activity in each fraction was measured at 30 °C for 10 min in reactions containing 10 mM MnCl₂ and 50 µM MBP. Reaction aliquots were spotted on p81 phosphocellulose paper. Washes and radioactivity detection were performed as already described.

Kinetic parameters

To determine the kinetic parameters of the WT and mutant enzymes, kinase reactions were performed as described above with a matrix of increasing concentrations of ATP and MBP. Apparent $V_{\max}$ values were measured from non-linear least-squares fitting of the primary data to the Michaelis–Menten equation. We plotted $1/V_{\max}$ (app) against $1/[ATP]$ to determine the intercepts and slopes from each curve, which in turn were used to calculate the values of K_m (app) [23].

RESULTS

Vector construction and expression of the c-Kit cytoplasmic domain

The methylotrophic yeast *P. pastoris* was used as a host for kinase expression. We found this organism to have no detectable background tyrosine phosphorylation, making it a very appropriate host for our studies [24]. By using an oligonucleotide linker that coded for the eight-residue Flag epitope [22] and a tri-glycine linker added for flexibility, the cDNA species of the cytoplasmic domain for WT and mutant c-Kit were cloned into the integrating *P. pastoris* expression vector pHL-D2 (Figure 1). The region of the vector encoding the linker and c-Kit insert was verified by sequencing. Linearized vector was then transformed into yeast spheroplasts and homologous recombinants were selected (Figure 1B). As described in the Experimental section, recombinant protein expression after induction was analysed over a 6-day period. Total cellular proteins from transfected and control cells were analysed by SDS/PAGE and Western blotting with the M2 anti-Flag monoclonal antibody. An immunoreactive

Figure 1 Structure of c-Kit catalytic domain expression cassette

(A) The Flag epitope with initiating methionine residue (MDYKDDDDKA) was fused with a tri-glycine linker to the coding portion of the c-Kit cytoplasmic domain. The W⁴² (DN790) and DY814 mutations are indicated. (B) Structure of the integrating expression vector pLL701. Striped regions indicate regions of identity that undergo homologous recombination.

Figure 2 Expression and tyrosine phosphorylation in yeast lysates transfected with WT, W⁴² and DY814 constructs

Yeast lysates were separated by SDS/PAGE and blotted with anti-Flag (A) or anti-phosphotyrosine (B) antibodies. All lanes contain equally loaded volumes of clone 18, WT, W⁴² and DY814 lysates respectively. Flag BAP refers to Flag-bacterial alkaline phosphatase used as a positive control that coincidentally migrates at the same molecular mass as the kinase. The positions of molecular mass markers are indicated (in kDa) at the left.

Figure 3 Western blotting and silver-staining analysis of purified kinase domains

(A) WT; (B) DY814. Approx. 10 ng of each purified kinase was subjected to SDS/PAGE and analysed by Western blotting with anti-FLAG (α-Flag) antibodies or by silver staining. The positions of molecular mass markers are indicated (in kDa) at the left.

protein with a molecular mass of approx. 50 kDa, the predicted molecular mass of the recombinant kinase, was observed in cells transformed with the expression vectors, but was absent from c-Kit-negative control cells. The optimal yield of kinase was obtained when cultures were lysed on day 4 after induction.

Tyrosine phosphorylation in WT, W⁴² and DY814 yeast lysates

We examined the state of kinase activation for the c-Kit catalytic domain of (1) WT, (2) W⁴², a naturally occurring kinase-inactive mutant [25], and (3) the DY814 mutant in yeast cells. After induction, lysates were prepared, resolved by SDS/PAGE and analysed by Western blotting with both M2 anti-Flag and anti-phosphotyrosine antibodies. As shown in Figure 2(A), at equal volume loadings the expression levels of the WT kinase were higher than those of the W⁴² and DY814 mutants. The WT enzyme was expressed at approx. 10-fold the level of the DY814 kinase. This difference in expression level was not related to the

Figure 4 Autophosphorylation of purified WT and DY814 proteins

Purified WT and DY814 proteins were incubated in the absence (–ATP) or presence (+ATP) of 1 mM ATP for 10 min, then analysed by SDS/PAGE and Western blotting with anti-phosphotyrosine antibodies. Vanadate, when added, was at 0.2 mM. The positions of molecular mass markers are indicated (in kDa) at the left.

genotype of the kinase because other DY814 clones generated showed higher levels of expression.

Anti-phosphotyrosine blotting (Figure 2B) revealed that, as expected, lysates from yeast cells expressing the W⁴² catalytic domain showed no detectable phosphotyrosine content (lane 2). Similarly, no phosphotyrosine content was observed in WT lysates (Figure 2B, lane 1). In contrast, extensive tyrosine phosphorylation of numerous proteins was observed in lysates prepared from *Pichia* cells expressing the DY814 catalytic domain (Figure 2B, lane 3) despite lower levels of DY814 protein. This observation indicates that the DY814 mutation alters the kinase to allow constitutive activation and extensive substrate phosphorylation in yeast cells.

Affinity purification of catalytic domains with M2 anti-Flag beads

Affinity purification of the soluble protein was performed with M2 anti-Flag beads as described in the Experimental section. To assess the efficiency and purity of the purification procedure, purified fractions were analysed by SDS/PAGE followed by Western blotting and silver staining. As shown in Figure 3, a 50 kDa band, corresponding to the recombinant c-Kit catalytic domain, was present in the Western blot and in silver-stained gels. An additional 47 kDa band was observed by anti-Flag Western blotting (Figure 3A) in some, but not all, preparations. This was probably due to C-terminal degradation because the Flag epitope, which is recognized by the blotting antibody, is at the N-terminus. The results indicate that the final product (50 and 47 kDa bands) was approx. 90% pure.

Autophosphorylation state of purified kinase domains

The DY814 mutation altered the activity of c-Kit in yeast cells, causing constitutive activation and tyrosine phosphorylation of several yeast proteins. However, the phosphorylation state of the kinase itself could not be determined from the Western blotting analysis of crude lysates owing to the presence of yeast proteins with similar molecular masses. Therefore we determined the phosphorylation state of the WT and DY814 enzymes after purification. The purification procedure was performed in the presence or absence of vanadate, to control for the possible presence of phosphatases. As shown in Figure 4 (lane 2), in the absence of vanadate, purified WT protein contained no detectable phosphotyrosine content, which is consistent with the lack of detectable phosphotyrosine in crude lysates. In the absence of

Table 1 Kinetic parameters for WT and DY814 kinases

Kinetic values were estimated from Lineweaver–Burk analyses of inverse plots.

Kinase	Specific activity at 20 nM (pmol/min per μ g)	K_m^{ATP} (μ M)	K_m^{MBP} (μ M)
WT	0.12	21.6	6.0
DY814	1.2	2.4	5.2

vanadate, purified DY814 protein also contained no detectable phosphotyrosine content (Figure 4, lane 4). This is in contrast with the extensive tyrosine phosphorylation of proteins observed in the lysates. However, when either the WT or mutant protein was purified in the presence of vanadate, autophosphorylated protein was detected in both cases (Figure 4, lanes 1 and 3). This observation therefore indicates that the WT and mutant enzymes are capable of autophosphorylation but that *Pichia*-derived phosphatases can remove those phosphates during the purification procedure.

When provided with ATP, both purified unphosphorylated enzymes were capable of autophosphorylation. Equal concentrations of purified proteins were incubated with ATP in a kinase reaction *in vitro*, resolved by SDS/PAGE, then blotted with anti-phosphotyrosine antibodies. Both kinases became reactive with anti-phosphotyrosine antibodies after the kinase reaction *in vitro*

(Figure 4, lanes 6 and 8). Densitometry measurements indicated that the DY814 mutant seemed to have a 2–3-fold enhanced level of autophosphorylation when as compared to with the WT.

Enzymic properties of c-Kit catalytic domains

To determine the efficiency of kinase-mediated substrate phosphorylation, the enzymes were incubated with ATP and MBP, a commonly used exogenous substrate in kinase reactions *in vitro*. A time course of phosphorylation revealed that the incorporation of phosphate was linear for the first 12 min (results not shown). Therefore maximal specific activity and K_m values were determined for ATP and MBP after 10 min incubations. Table 1 summarizes these results. The apparent K_m^{ATP} for the DY814 mutant was one-tenth that of the WT; however, the DY814 K_m^{MBP} was similar to that of WT. At enzyme concentrations of 20 nM each, the DY814 mutant displayed a maximal specific activity that was 10-fold that observed for the WT. These results indicate that the mutant enzyme has a higher catalytic activity and a higher affinity for ATP. These differences might result in a more potent kinase *in vitro* and potentially *in vivo*.

Concentration dependence of soluble kinase activity

Autophosphorylation of receptor tyrosine kinases is thought to occur by transphosphorylation within dimers or oligomers [5]. However, intramolecular phosphorylation can also occur. To distinguish between these two possibilities, we used both filter-

Figure 5 Activity dependence on enzyme concentration: SDS/PAGE analysis

Purified WT or DY814 enzyme was incubated with radioactive ATP for 10 min at 30 °C. Reaction products were analysed by SDS/PAGE, autoradiography and PhosphorImager analysis. (A, B) Autoradiography of serially diluted WT (A) and DY814 (B) enzymes; (C, D) PhosphorImager analysis of gels in (A) and (B) respectively. Each lane in the gels (A, B) corresponds to one point on the graphs (C, D). All curves are the least-squares best fit to a quadratic equation.

Figure 6 Activity dependence on enzyme concentration: filter-binding assay

Purified WT (●) or DY814 (○) enzyme was incubated with [γ - 32 P]ATP for 10 min at 30 °C. The incorporation of ATP was determined by using the phosphocellulose binding assay as described in the Experimental section. Both curves are least-squares fits to a linear-quadratic equation.

plate binding assays and kinase reactions *in vitro* followed by SDS/PAGE analysis and autoradiography of serially diluted kinase to measure the dependence of kinase activity on enzyme concentration. Increased activity/ μ g at higher enzyme concentrations would indicate that the spontaneous formation of dimers or oligomers activates the enzyme. As shown in Figures 5(A) and 5(C), analyses by autoradiography (Figure 5A) and PhosphorImager analysis (Figure 5C) indicated that WT activity increased with increasing kinase concentration in a non-linear fashion. This hyperbolic curve of activity was also observed in the filter-plate assay measuring autophosphorylation (Figure 6). This result is consistent with a requirement for trans-phosphorylation in autophosphorylation of the WT enzyme and also indicates that the enzymic activity associated with kinase dimers or oligomers increases.

Analysis of the DY814 mutant yielded strikingly different results. At similar levels of activity to those of the WT enzyme (at one-fifth the enzyme concentrations), we observed a primarily linear dependence of kinase autophosphorylation activity (Figures 5B and 5D), indicating that DY814 autophosphorylation was not concentration-dependent. By using the filter-plate assay over a similar enzyme concentration range as with the WT, the same linear curve was observed for autophosphorylation (Figure 6). These results indicate that the DY814 mutation either promotes dimerization for autophosphorylation or enables the kinase to autophosphorylate itself in an intramolecular manner.

Effect of bivalent anti-Flag antibody on autophosphorylation

The non-linear dependence on enzyme concentration observed for WT activity suggests a requirement for enzyme dimerization or oligomerization for activation. We took advantage of the Flag epitope at the N-terminus of the kinase domains to observe the effect of bivalent anti-Flag antibodies on activity. Purified kinase domains (approx. 6 nM) were incubated with increasing concentrations of M2 anti-Flag antibodies for 1 h at room temperature. After antibody binding, kinase reactions were performed and analysed by SDS/PAGE and PhosphorImager quantification. As shown in Figure 7(A), optimal concentrations of antibody enhanced WT autophosphorylation 2.7-fold. However, with

Figure 7 Effect of anti-Flag antibodies on autophosphorylation

Purified WT (A) or DY814 (B) enzymes were nutated with various concentrations of anti-Flag antibodies for 1 h at room temperature. Kinase reactions were performed as described in the Experimental section. Reactions were analysed by SDS/PAGE, autoradiography and PhosphorImager analysis.

further increases in antibody concentration, autophosphorylation decreased to levels lower than those observed in the absence of antibody. This result is consistent with the hypothesis that bivalent M2 antibody stabilizes dimers of catalytic domains. At concentrations at which antibody is in excess, antibody binding to single domains would be favoured, resulting in the disruption of dimers.

Anti-Flag antibodies affected DY814 kinase activity in a different manner (Figure 7B). When antibody concentrations similar to those observed to be optimal for the WT enzyme were added, only a minimal increase in autophosphorylation was detected. With further increases in antibody concentration, the activity decreased to 26% of that observed in the absence of antibody. This result is consistent with the pre-existence of DY814 dimers that can be disrupted by high concentrations of anti-Flag antibody. Alternatively, the presence of excess antibody might have a steric effect on the activity of the kinase or induce cross-linked aggregates of dimers with impaired activity.

Gel-filtration analysis of WT and DY814 kinase domains

The dependence on enzyme concentration and anti-Flag mediated activation for the WT kinase was consistent with an

Figure 8 Gel-filtration analysis of WT (●) and DY814 (○)

Purified protein was chromatographed on a Sephacryl S-200 column and fractions were measured for kinase activity as described in the Experimental section. The column was calibrated with molecular mass standards (as indicated above the elution profiles) immediately before the samples were analysed.

enzyme requiring dimerization or oligomerization for transphosphorylation and activation. In contrast, the DY814 mutant did not depend on enzyme concentration for activity, nor was it activated by anti-Flag antibodies. The mutation might therefore promote stable dimer formation. To test this possibility, we analysed the behaviour of WT and DY814 on a gel-filtration column. Affinity-purified protein was applied to a Sephacryl S-200 column controlled by an FPLC apparatus. Fractions were eluted with buffer A (described in the Experimental section) and measured for kinase activity. As shown in Figure 8, WT activity was detected in a single peak corresponding to a molecular mass of 50 kDa. In contrast, DY814 kinase activity was detected in a peak corresponding to a molecular mass of 100 kDa. Therefore, whereas the WT kinase migrated primarily as a monomer, the DY814 mutant seemed to migrate as a dimer.

DISCUSSION

We have evaluated the kinetic properties of purified WT and DY814 c-Kit catalytic domains. Lysates from yeast cells expressing DY814 were found to contain numerous tyrosine-phosphorylated proteins, whereas lysates from yeast cells expressing the WT contained no detectable phosphotyrosine. We found that the DY814 mutant had an increased apparent affinity for ATP and a higher specific activity compared with those of the WT kinase at concentrations up to 100 nM. Whereas DY814 autophosphorylation was primarily linearly dependent on enzyme concentration, WT autophosphorylation was non-linear. Increasing concentrations of anti-Flag antibody enhanced, and then inhibited, WT kinase activity but had primarily an inhibitory effect on DY814. Finally, we found that the WT kinase migrates as a monomer, whereas DY814 migrates as a dimer on a gel-filtration column. In summary, our experiments indicate that the active form of both enzymes is a dimer. However, dimerization

is transient in the WT protein, whereas the DY814 dimer is stable.

Our approach to addressing mechanistic differences between the two enzymes involved analysing the properties of purified recombinant proteins expressed in the yeast organism *P. pastoris*. This organism was chosen because it does not have measurable endogenous tyrosine phosphorylation [24]. The advantages of this system include proper protein processing and folding in a eukaryotic environment, and ease of manipulation. Our initial attempt to use bacteria for expression was hampered by difficulties in detecting kinase activity [26].

The fact that both WT and DY814 were capable of autophosphorylation after purification indicates that pre-phosphorylation of the kinases is not required for either WT or DY814 activity. However, our results do not address the question of the effect of kinase phosphorylation on c-Kit activity. Autophosphorylation has been demonstrated to increase the activity of the epidermal growth factor, insulin and insulin-like growth factor-1 receptors [27–31]. It is therefore possible that autophosphorylation also modulates the kinase activity of c-Kit.

Several research groups have measured the enzymic parameters for the epidermal growth factor receptor [32–35]. The K_m^{ATP} values that we obtained for the WT and mutant c-Kit catalytic domains fall within the range of these measurements. The specific activity that we obtained for the DY814 kinase is 10-fold that found for the WT kinase at an enzyme concentration of 20 nM. These values are probably underestimates because the active fraction of enzyme is not known. A more accurate determination of the active fraction would allow a better estimate of the V_{max} for the enzymes.

The observation that WT activity displays a non-linear dependence on enzyme concentration is consistent with a requirement for dimerization or oligomerization for activity. The fact that anti-Flag antibodies enhance and then inhibit WT activity at increasing concentrations supports this hypothesis. The co-expression of WT c-Kit with kinase-inactive receptors results in a dominant-negative phenotype [4]. Because our results indicate that the active form of the kinase is a dimer, heterodimers of WT and inactive receptors would be expected to have decreased activity and therefore less signalling capacity.

One effect of the DY814 mutation is the promotion of stable dimers. We demonstrated that DY814 has primarily a linear activity dependence on enzyme concentration, indicating that further aggregation is not required for autophosphorylation or substrate phosphorylation. The lack of increased activity observed when anti-Flag antibody was added is also consistent with pre-existing dimers. Lastly, gel-filtration analysis of purified DY814 protein showed that the active fraction migrated through the column as a dimer. In Ba/F3 cells transfected with the DY814 holoreceptor, chemical cross-linking agents could detect c-Kit dimers only in the presence of ligand [15]. However, bis(sulphosuccinimidyl) suberate, a water-soluble cross-linker that does not penetrate the cell, was used in this analysis, so only the association of the extracellular domains would be detected. It is therefore possible that the dimerizing effect of the mutation on c-Kit catalytic domains also occurs with the holoreceptor.

Although the three-dimensional structure of the c-Kit kinase domain is not known, it is similar in sequence to the fibroblast growth factor receptor and the insulin receptor, whose crystal structures have been determined [36,37]. These structures indicate that Asp-647 of the fibroblast growth factor receptor and Asp-1156 of the insulin receptor (residues equivalent to Asp-814 in c-Kit) are found in the activation loop located between the ATP-binding and substrate phosphorylation lobes [36]. In the inactive form, this activation loop blocks access to the substrate-binding

site of both receptors. If Asp-814 occupies a similar location in the c-Kit receptor, then this mutation might alter conformation of the activation loop, promoting dimerization, transphosphorylation and activation. Our results indicate that another effect of the DY814 mutation is to increase the apparent affinity for ATP. This might also be due to alterations in the conformation of the activation loop by the mutation.

The normal development and survival of mast cells is controlled primarily by c-Kit and its ligand, steel factor [4]. c-Kit is also expressed in a variety of other cells, including melanocytes, germ cells and haemopoietic stem cells [38]. c-Kit has also been found in tumours derived from cells that do not normally express this receptor [39–42]. Therefore the activation of c-Kit mutations might contribute to the oncogenic transformation of many cell types. Furthermore, because Asp-814 is highly conserved in other receptor tyrosine kinases including the platelet-derived growth factor, colony-stimulating factor 1, insulin and hepatocyte growth factor receptors [43], substitutions at this residue might also result in the constitutive activation and transforming ability of other receptors, possibly through constitutive dimerization.

Our observation that purified soluble kinase domains reproduce the behaviour of both the WT and DY814 c-Kit receptors expressed in cells indicates that this approach *in vitro* is effective in determining the effects of activating mutations on receptor tyrosine kinases. Further structural and biochemical analysis of the WT and DY814 c-Kit tyrosine kinases should be extremely useful for understanding the role of dimerization in kinase activation.

We thank A. Bernstein and P. Besmer for supplying us with DNA clones, P. Yip for technical assistance, and D. Isenman and R. Rottapel for their helpful comments on the manuscript. This work was supported by a grant from the National Cancer Institute of Canada (to S. A. B.).

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Received 6 July 1998/4 November 1998; accepted 26 November 1998