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Signal transduction by normal isoforms and *W* mutant variants of the Kit receptor tyrosine kinase

Alastair D.Reith[1,4], Christine Ellis[1,5], Stewart D.Lyman[3], Dirk M.Anderson[3], Douglas E.Williams[3], Alan Bernstein[1,2] and Tony Pawson[1,2]

[1]Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute of Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5, [2]Department of Molecular and Medical Genetics, University of Toronto, Canada and [3]Immunex Corporation, 51 University Street, Seattle, WA 98101, USA.

Present addresses: [4]Ludwig Institute for Cancer Research, 91 Riding House Street, London W1P 8BT and [5]Institute for Cancer Research, Chester Beatty Laboratories, Fulham Road, London SW3 6JB, UK

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Germline mutations at the *Dominant White Spotting* (*W*) and *Steel* (*Sl*) loci have provided conclusive genetic evidence that c-*kit* mediated signal transduction pathways are essential for normal mouse development. We have analysed the interactions of normal and mutant *W*/c-*kit* gene products with cytoplasmic signalling proteins, using transient c-*kit* expression assays in COS cells. In addition to the previously identified c-*kit* gene product (Kit+), a second normal Kit isoform (KitA+) containing an in-frame insertion, Gly-Asn-Asn-Lys, within the extracellular domain, was detected in murine mast cell cultures and mid-gestation placenta. Both Kit+ and KitA+ isoforms showed increased autophosphorylation and enhanced association with phosphatidylinositol (PI) 3′ kinase and PLCγ1, when stimulated with recombinant soluble Steel factor. No association or increase in phosphorylation of GAP and two GAP-associated proteins, p62 and p190, was observed. The two isoforms had distinct activities in the absence of exogenous soluble Steel factor; Kit+, but not KitA+, showed constitutive tyrosine phosphorylation that was accompanied by a low constitutive level of association with PI-3′ kinase and PLCγ1. Introduction of the point substitutions associated with W^{37} (Glu582 → Lys) or W^{41} (Val831 → Met) mutant alleles into c-*kit* expression constructs abolished (W^{37}) or reduced (W^{41}) the Steel factor-induced association of the Kit receptor with signalling proteins in a manner proportional to the overall severity of the corresponding *W* mutant phenotype. These data suggest a diversity of normal Kit signalling pathways and indicate that *W* mutant phenotypes result from primary defects in the Kit receptor that affect its interaction with cytoplasmic signalling proteins.

Key words: mouse development/Kit isoforms/signal transduction/*Steel* locus/*W* locus

Introduction

Growth factor receptors with intrinsic tyrosine kinase activity represent a major class of proteins that serve to regulate cellular proliferation in eukaryotes. In well studied prototype receptor tyrosine kinases (RTKs), interaction of the extracellular domain with specific ligands stimulates receptor dimerization, transient activation of the tyrosine kinase activity within the intracellular domain of the RTK protein and autophosphorylation. In turn, this leads to the formation of complexes with cytoplasmic signalling proteins that serve to transduce the signal towards the nucleus, so facilitating an appropriate cellular response to growth factor and hormone-mediated fluctuations in a cell's microenvironment (see Ullrich and Schlessinger, 1990; Koch *et al.*, 1991 for recent reviews).

A number of the proteins that associate with activated RTKs, such as βPDGFR, EGFR, CSF-1R and insulin receptor have been identified. These substrates include phospholipase Cγ1 (PLCγ1) (Downing *et al.*, 1989; Meisenhelder *et al.*, 1989; Morrison *et al.*, 1990; Margolis *et al.*, 1989, 1990a), phosphatidylinositol 3′ kinase activity (Kaplan *et al.*, 1987; Varticovski *et al.*, 1989; Bjorge *et al.*, 1990; Endemann *et al.*, 1990; Reedijk *et al.*, 1990; Ruderman *et al.*, 1990; Shurtleff *et al.*, 1990), GTPase activating protein (GAP) (Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990) and the src family of tyrosine kinases (Kypta *et al.*, 1990). These proteins all contain SH2 domains, which apparently direct their interaction with autophosphorylated sites in activated receptors (Anderson *et al.*, 1990a; Moran *et al.*, 1990). Increased phosphorylation and activation of the serine/threonine kinase, c-Raf-1, in response to ligand activation of βPDGFR, CSF-1R and EFGR has also been demonstrated (Morrison *et al.*, 1988, 1989; Baccarini *et al.*, 1990; App *et al.*, 1991).

The role of RTK-mediated signalling pathways in directing cellular responses to environmental change suggests that components of such pathways may be critical for normal mammalian developmental processes. The ability of a cell to respond to an external signal is determined firstly by the specificity of growth factor−receptor interactions, and the restricted expression of genes encoding these proteins during development (Adamson, 1987; Mercola and Stiles, 1988; Rappolee *et al.*, 1988; Wilkinson *et al.*, 1988; Regenstreif and Rossant, 1989; Klein *et al.*, 1990a; Martin-Zanca *et al.*, 1990; Matsui *et al.*, 1990; Mercola *et al.*, 1990; Orr-Urtreger *et al.*, 1990). Additional levels of regulation and diversity can be achieved by expression of polymorphic forms of ligands and/or receptor tyrosine kinases resulting from alternative transcriptional initiation (Vu *et al.*, 1989; Rathjen *et al.*, 1990), differential RNA processing (Ladner *et al.*, 1987; Klein *et al.*, 1990b; Petch *et al.*, 1990; Reid *et al.*, 1990; Middlemas *et al.*, 1991), or post-translational processing (Rettenmier and Roussel, 1988) of products of a single locus, or by formation of heterodimers of closely related proteins, as seen for PDGF (see Heldin and Westermark, 1989 for review). It is also apparent that different activated RTKs interact with distinct and characteristic subsets of potential intracellular substrates,

providing another mechanism for establishing the specificity of RTK signalling pathways. For example, GAP and PLCγ are major substrates for activated forms of βPDGFR and EGFR (Downing *et al.*, 1989; Margolis *et al.*, 1989, 1990a,b; Meisenhelder *et al.*, 1989; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990), but not the CSF-1 receptor (Downing *et al.*, 1989; Reedijk *et al.*, 1990).

Genetic evidence for the roles played by RTK-mediated signalling pathways in mammalian development is sparse (Pawson and Bernstein, 1990). One of the few examples is provided by strains of mice bearing mutant alleles at the *Dominant White Spotting (W)* (Little, 1915) or *Steel (Sl)* (Sarvella and Russell, 1956) loci. Mutations at the *W* locus have been attributed to loss of function mutations in the c-*kit* receptor tyrosine kinase (Chabot *et al.*, 1988; Geissler *et al.*, 1988; Nocka *et al.*, 1989; 1990a; Reith *et al.*, 1990; Tan *et al.*, 1990), whilst *Sl* mutants have been found to bear mutations in a Kit ligand, variously termed mast cell growth factor (Anderson *et al.*, 1990b; Copeland *et al.*, 1990; Williams *et al.*, 1990), stem cell factor (Martin *et al.*, 1990; Zsebo *et al.*, 1990a,b) or Kit ligand (Huang *et al.*, 1990; Nocka *et al.*, 1990b), referred to here as Steel factor, that can exist in soluble or membrane bound forms. The identical phenotypes induced by *W* or *Sl* mutations and the complementary sites of action of these mutations in stem cells and their microenvironment, respectively (Russell, 1979; Silvers, 1979), provide elegant genetic evidence that the Kit signalling pathway is essential in mediating appropriate cell–cell interactions in the development of hematopoietic, germ cell and melanogenic lineages.

In this report, we have used transient gene expression assays in COS cells to investigate the biochemical responses of normal and *W* mutant recombinant isoforms of Kit to recombinant soluble Steel factor. A second normal Kit isoform was found to be co-expressed with that previously reported in mast cell cultures. These normal Kit isoforms display distinct signalling properties, suggesting a diversity of Kit signalling pathways that may provide additional specificity of response to particular environmental signals. We also show that introduction of single amino acid substitutions, associated with severe or mild *W* mutant phenotypes, into Kit proteins confers deficiencies in the Kit signalling pathway proportional to the overall severity of the corresponding *W* mutant phenotype.

Results

Identification of a second Kit isoform

In the course of identification of point substitutions in the c-*kit* coding sequence in *W* mutant mice (Reith *et al.*, 1990), a 12 bp insertion between amino acid residues 512 and 513 in the published murine c-*kit* cDNA sequence (Qui *et al.*, 1988) was found in a number of cDNA clones, irrespective of *W* genotype or mRNA source (Figure 1). This asparagine-rich insert (termed NRI) has the capacity to encode an additional four amino acids, Gly-Asn-Asn-Lys, within the extracellular domain of the Kit receptor (Figure 1). The isolation of both Kit isoforms in homogenous cell populations of fetal or adult mast cell cultures suggests that both forms are co-expressed in at least some cell types. Interestingly, the NRI is present in the published human c-*kit* cDNA sequence (Yarden *et al.*, 1987) and we have found both Kit isoforms to be expressed in human glioblastoma and erythroleukemic cell lines (data not shown).

Glu582 → Lys and Val831 → Met point mutations confer Kit protein phenotypes characteristic of W^{37} or W^{41} mutant mice

Animals homozygous for the severe W^{37} or mild W^{41} mutant alleles are characterized by the expression of normal levels of immature Kit proteins that either lack, or are deficient in, *in vitro* kinase activity, respectively. The 160 kDa glycosylated form of Kit in W^{37} mutant cells is unstable and has no *in vitro* kinase activity, whilst the W^{41} mutant form of Kit has normal stability but decreased *in vitro* kinase activity (Nocka *et al.*, 1990a; Reith *et al.*, 1990). It has been proposed that these defects result from Glu582 → Lys and Val831 → Met point substitutions identified in c-*kit* cDNA clones isolated from W^{37} and W^{41} animals, respectively (Nocka *et al.*, 1990a; Reith *et al.*, 1990).

Primary mast cell cultures provide a useful *in vitro* model

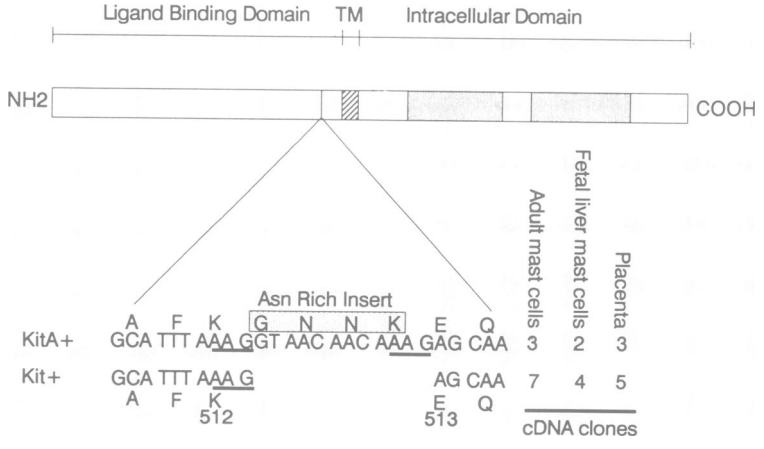

Fig. 1. Identification of a second isoform of the Kit receptor. The identity and location of the asparagine-rich insert (NRI) within the ligand binding domain of the Kit polypeptide is illustrated. The 12 nucleotide insertion, encoding Gly-Asn-Asn-Lys, between amino acids 512 and 513 in the published murine c-*kit* cDNA sequence (Qui *et al.*, 1988), was identified in a number of c-*kit* cDNA clones generated from mRNA of mast cells derived from adult marrow or 15 day gestation fetal liver and 15 day gestation placenta. The non-NRI isoform, Kit+, was found to be the more abundant mRNA species in these cell types. Consensus 5′ splice junction sequences AAG flanking NRI are underlined. Hatched and shaded areas indicate the single transmembrane domain and split kinase domain of the Kit polypeptide, respectively. (A) alanine; (E) glutamate; (F) phenylalanine; (G) glycine; (K) lysine; (N) asparagine; (Q) glutamine.

for cellular and molecular analyses of *W* mutant phenotypes and Kit signalling in a physiologically relevant cell type. However, the expression of endogenous Kit proteins, and their refractiveness to DNA transfection procedures, makes them unsuitable for analysis of the biochemical consequences of specific experimentally-induced mutations in components of the Kit signalling pathway. In contrast, the SV40-transformed monkey fibroblast cell line COS-1 (Gluzman, 1981) provides an easily manipulable *in vitro* culture system in which to analyse recombinant protein expression. COS cells do not express endogenous Kit proteins and are unable to support mast cell growth in co-culture assays (Anderson *et al.* 1990b; L.Forrester and A.D.Reith, unpublished observations), and so were expected to provide a neutral background in which to analyse Steel factor/Kit signalling. To this end, the NRI polymorphism, W^{37} Glu582 → Lys or W^{41} Val831 → Met point substitutions were introduced into SV40-murine c-*kit* cDNA expression constructs (Figure 2A) for use in transient gene expression assays in COS cells.

As seen in Figure 2B, immunoprecipitation of cell lysates prepared from COS cell transfectants with Kit antisera (Reith *et al.*, 1990) indicated that all recombinant c-*kit* constructs encoded similar levels of native non-glycosylated 106 kDa Kit protein. Whilst equivalent levels of the differentially glycosylated 124 kDa and 160 kDa forms of Kit were observed with the pKit+, pKitA+ and pKitA41 cDNA

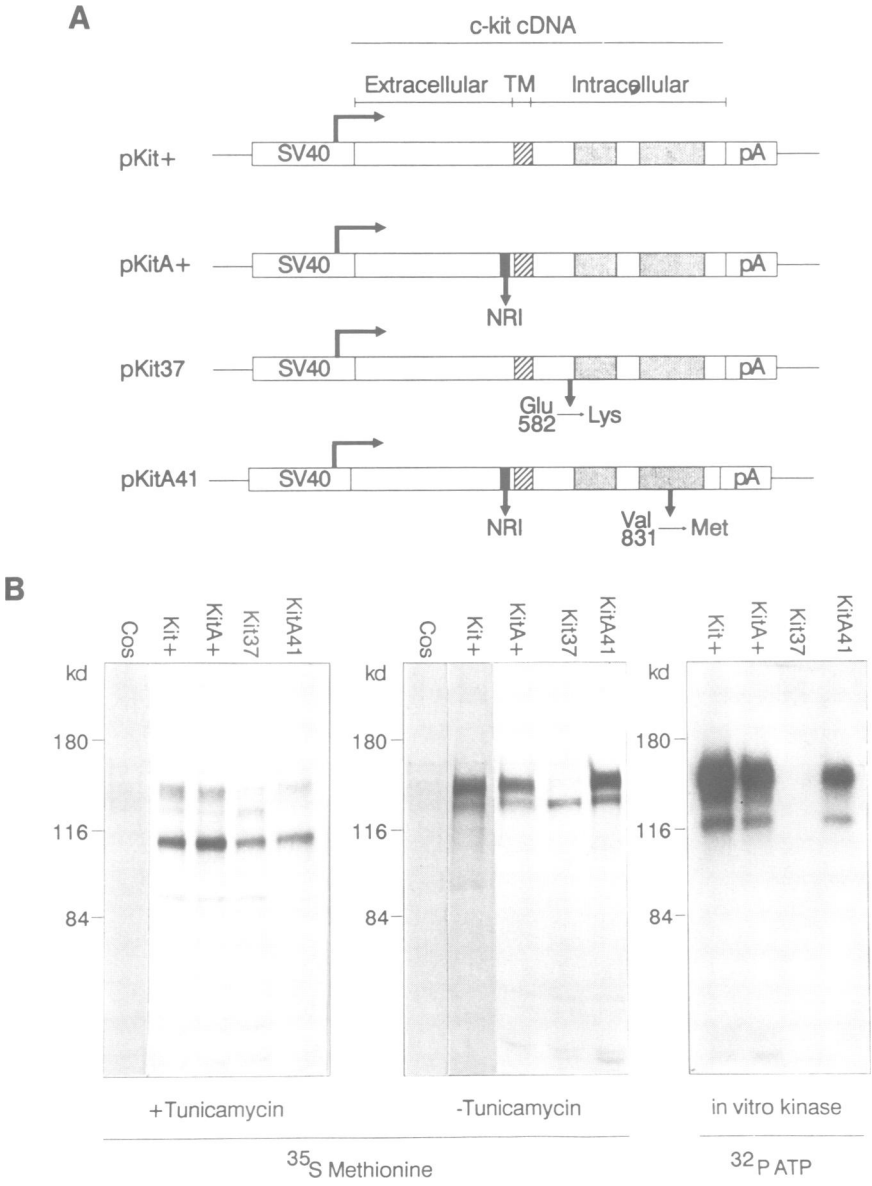

Fig. 2. Point substitutions in c-*kit* identified in *W* mutant animals confer deficiencies in Kit kinase activity. (**A**) Gene expression constructs. All constructs were made in the SV40-based expression vector pECE (Ellis *et al.*, 1986). pKit+ and pKitA+ both contain a full length normal murine c-*kit* cDNA, differing only in the absence and presence, respectively, of NRI (black box). pKit37 differs from pKit+ only in the presence of a GC → AT point mutation, resulting in Glu582 → Lys substitution associated with W^{37} mutant c-*kit* allelle. pKitA41 differs from pKitA+ only in the presence of a GC → AT mutation that confers the Val831 → Met point substitution identified in the W^{41} mutant c-*kit* allele. Hatched and shaded areas indicate the location of the single transmembrane domain and split kinase domain of Kit, respectively. (**B**) Protein phenotypes of recombinant Kit isoforms and point mutants. Normal (Cos) or pKit+, pKitA+, pKit37 or pKitA41 COS cell transfectants were metabolically labelled with [^{35}S]methionine in the presence or absence of 500 μg/ml tunicamycin where appropriate, lysed, immunoprecipitated with Kit antisera and subjected to 7.5% SDS–PAGE prior to autoradiography. For *in vitro* kinase assays, immunoprecipitates were incubated in the presence of [γ-^{32}P]ATP prior to SDS–PAGE and autoradiography.

expression constructs, the Glu582 → Lys mutation resulted in reduced steady-state levels of the 160 kDa Kit glycosylated form, similar to that seen in W^{37} homozygous mast cell cultures (Nocka *et al.*, 1990a; Reith *et al.*, 1990), suggesting that this substitution destabilizes the mature Kit protein.

Both the 124 kDa and 160 kDa forms of Kit+ and KitA+ had high levels of *in vitro* kinase activity. In contrast, the Glu582 → Lys mutation of pKit37 abolished such activity (Figure 2B), again characteristic of Kit proteins synthesized by W^{37} homozygous mast cell cultures (Nocka *et al.*, 1990a; Reith *et al.*, 1990). Introduction of the Val831 → Met point substitution conferred a quantitative reduction in Kit *in vitro* kinase activity relative to either normal Kit isoform (Figure 2B), a result comparable to that observed for Kit in W^{41}/W^{41} mast cell cultures (Nocka *et al.* 1990a; Reith *et al.*, 1990).

Signal transduction properties of normal and W mutant Kit isoforms

To investigate the consequences of the NRI polymorphism and W^{37} and W^{41} point mutations on the Kit signal transduction pathway, we determined the phosphorylation status of recombinant Kit proteins, and their association with a variety of candidate targets, in response to recombinant soluble Steel factor.

Autophosphorylation. Stimulation of the autophosphorylation activity of receptor tyrosine kinases is an immediate response to ligand stimulation (Ullrich and Schlessinger, 1990). To investigate the response of normal and *W* mutant forms of Kit to soluble Steel factor, COS cell lysates prepared from untreated or stimulated transfectants were immunoprecipitated with antisera to the Kit receptor and subjected to Western blot analysis using affinity purified anti-phosphotyrosine antibodies (Letwin *et al.*, 1988).

As shown in Figure 3A, tyrosine phosphorylation of the KitA+ isoform was apparent only after treatment of COS cell lysates with soluble Steel factor. In contrast, the Kit+ isoform exhibited constitutive levels of tyrosine phosphorylation that increased on Steel factor stimulation. Interestingly, the extent of this constitutive phosphorylation varied according to the growth conditions of the COS cells, increasing when cultures were grown in low serum conditions for 24 or 40 h prior to assay (Figure 3B). Introduction of the W^{37} Glu582 → Lys point substitution into the Kit+ isoform abolished tyrosine phosphorylation of Kit either in the presence or absence of exogenous recombinant soluble Steel factor (Figure 3A). Similarly, the W^{41} Val831 → Met point substitution conferred a quantitative decrease in soluble Steel factor-inducible tyrosine phosphorylation levels of Kit (Figure 3A). Taken together, these data indicate that constitutive and Steel factor-inducible tyrosine phosphorylation of Kit is an intrinsic function of the autophosphorylation capacity of the Kit kinase domain.

In an attempt to identify other proteins, phosphorylated at tyrosine residues in response to Steel factor, whole cell lysates were prepared from COS cell transfectants treated with soluble Steel factor and subjected to western blot analysis using affinity purified anti-phosphotyrosine antibodies. As shown in Figure 4, increased tyrosine phosphorylation of a single protein species of ~160 kDa was apparent on stimulation of pKitA+ transfectants with Steel factor. Since the size and appearance of this protein in other COS cell transfectants was identical to that of Kit, we concluded that this protein is likely to be tyrosine

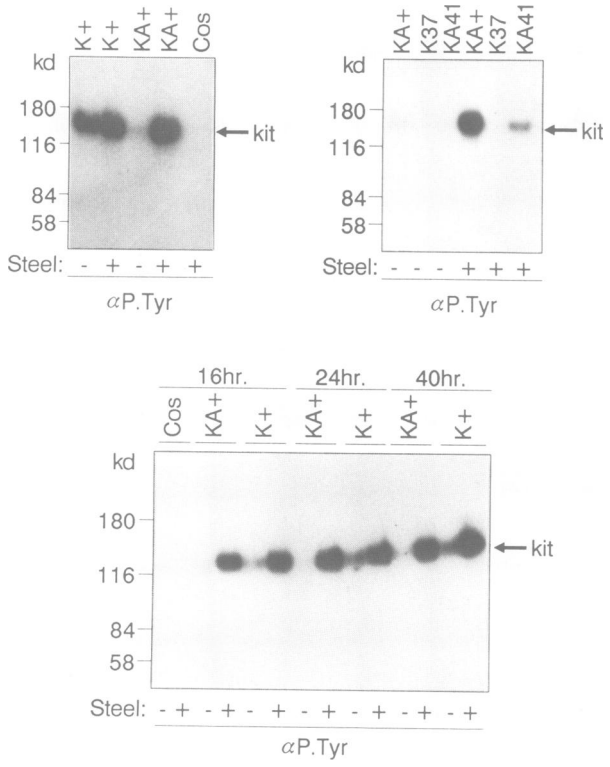

Fig. 3. Tyrosine phosphorylation of normal and *W* mutant Kit isoforms in response to recombinant soluble Steel factor. (**A**) Normal (Cos), or pKit+ (K+), pKitA+ (KA+), pKit37 (K37) or pKitA41 (KA41), COS cell transfectants were grown in low serum medium for 40 h prior to stimulation with 1 μg recombinant soluble Steel factor (Steel), where indicated (+). Whole cell lysates were immunoprecipitated with Kit antibody and subjected to Western blot anlaysis using anti-phosphotyrosine antibodies (αP.Tyr). (**B**) Normal (Cos), or pKit+ (K+) or pKitA+ (KA+) COS cell transfectants were grown in low serum medium for 16, 24 or 40 h, as indicated, prior to stimulation with 1 μg recombinant Steel factor (Steel), where indicated (+). Whole cell lysates were immunoprecipitated with Kit antisera and subjected to Western blot analysis using anti-phosphotyrosine antibodies (αP.Tyr).

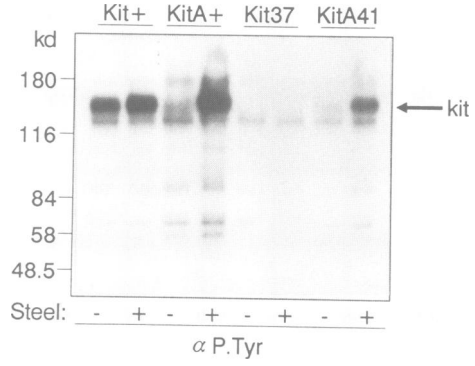

Fig. 4. Anti-phosphotyrosine Western blot analysis of COS cell transfectants. COS cells transfected with pKitA+, pKit37 or pKitA41 were grown in low serum medium for 40 h prior to stimulation with 1 μg recombinant soluble Steel factor (Steel) as indicated (+). Whole cell lysates were prepared and directly subjected to Western blot analysis using anti-phosphotyrosine antibodies (αP.Tyr).

phosphorylated Kit receptor. No increased tyrosine phosphorylation of any other protein was apparent on stimulation of transfectants with soluble Steel factor.

GTPase activating protein (GAP). GAP stimulates the intrinsic GTPase activity of $p21^{ras}$ proteins, thereby returning the active GTP bound form of $p21^{ras}$ to the inactive GDP bound form (McCormick, 1989). GAP associates with, and becomes tyrosine phosphorylated by, certain activated RTKs such as βPDGFR, and furthermore has been shown to associate with two proteins, p62 and p190, which also become tyrosine phosphorylated on receptor activation (Ellis *et al.*, 1990; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990). To assess any role for GAP and associated proteins in the Kit signal transduction pathway, lysates of COS cell transfectants were immunoprecipitated with GAP antisera (Ellis *et al.*, 1990) and the phosphorylation status of immuoprecipitated proteins determined by Western blot analysis using affinity purified anti-phosphotyrosine antibodies. No increase in tyrosine phosphorylation of GAP, p190 or p62 was apparent on Steel factor stimulation of COS cells expressing either normal or *W* mtuant isoforms of Kit, although increased tyrosine phosphorylation of these substrates was apparent on PDGF stimulation of βPDGFR COS cell transfectants, in v-src transfectants and in COS cells stimulated with EGF (Figure 5). Thus, it would appear that GAP and associated proteins are not major targets of the activated Kit kinase.

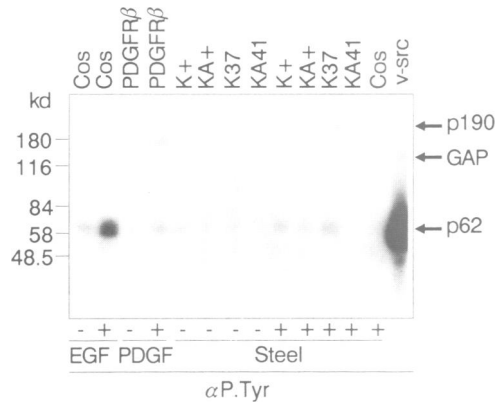

Fig. 5. GAP and GAP-associated p190 and p62 proteins are not major targets of Steel factor activated Kit. COS cells or COS cells transfected with pKitA+ (KA+), pKit37 (K37), pKitA41 (KA41), pmtPDGFR (PDGFR) or pECEv-src (v-src) were grown in low serum for 40 h prior to stimulation with 1 μg recombinant soluble Steel factor (Steel), 75 nM platelet-derived growth factor (BB homodimer) (PDGF) or 80 nM epidermal growth factor (EGF), as appropriate followed by lysis and immunoprecipitation with GAP antisera. Immunoprecipitates were then subjected to Western blot analysis using anti-phosphotyrosine antibodies (αP.Tyr). COS cells express endogenous epidermal growth factor receptor (data not shown).

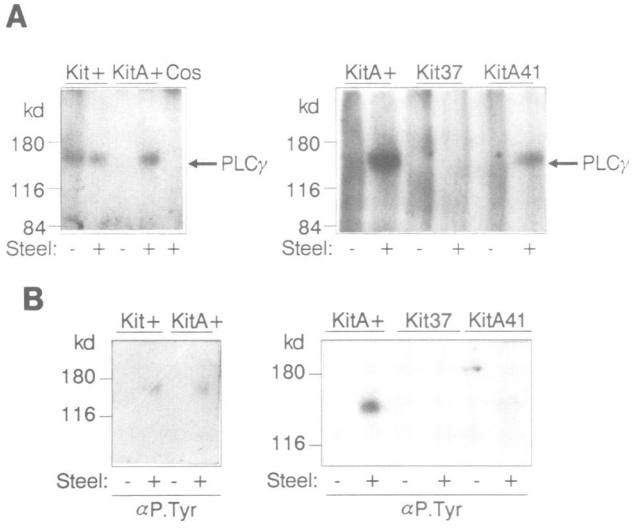

Fig. 6. Association of normal and *W* mutant Kit isoforms with PLCγ1 on Steel factor stimulation. (**A**) Normal (Cos) or COS cells transfected with pKit+, pKitA+, pKit37 or pKitA41 were grown in low serum for 40 h prior to stimulation with 1 μg recombinant soluble Steel factor (Steel). Whole cell lysates were immunoprecipitated with Kit antisera and subjected to Western blot analysis using PLCγ1 antibodies. (**B**) COS cell transfectants stimulated as in (A), were immunoprecipitated with PLCγ antisera and subjected to Western blot analysis using anti-phosphotyrosine antisera (αP.Tyr).

Phospholipase Cγ1 (PLCγ1). A second substrate utilized by some RTK mediated signalling pathways is phospholipase Cγ1, that associates with stimulated βPDGFR (Downing *et al.*, 1989; Meisenhelder *et al.*, 1989; Morrison *et al.*, 1990) and EGFR (Margolis *et al.*, 1989, 1990a,b). Such association is concomitant with an increase in tyrosine and serine phosphorylation of PLCγ1, modifications which may increase PLCγ1 catalytic activity (Nishibe *et al.*, 1990). To ascertain the potential role of PLCγ1 in Kit-mediated signal transduction, COS cell transfectants were immunoprecipitated with Kit antisera and subjected to Western blot analysis with affinity purified anti-PLCγ1 antisera (Decker *et al.*, 1990).

As shown in Figure 6A, PLCγ1 associated with Kit+ in the absence of exogenous soluble Steel factor, and with both Kit+ and KitA+ on Steel factor stimulation. In contrast, KitA41 exhibited reduced levels of association with PLCγ1 when stimulated with Steel factor, relative to either normal Kit isoform, and no association of PLCγ1 was detected with the kinase-inactive Kit37 mutant protein, either in the presence or absence of soluble Steel factor. When COS cell lysates were immunoprecipitated with anti-PLCγ1 antisera and subjected to Western blot analysis with anti-phosphotyrosine antibodies, we observed a tyrosine phosphorylated protein of ~150 kDa, detectable in pKit+ transfectants, but only on soluble Steel factor stimulation of KitA+ expressing cells. This protein was found to be less abundant in KitA41 transfectants, relative to normal Kit isoforms, and undetectable in Kit37 lysates (Figure 6B). This tyrosine phosphorylated protein may correspond to PLCγ1, although we cannot exclude the possibility that it represents PLCγ1-associated Kit.

Phosphatidylinositol-3'(PI-3) kinase activity. PI-3′ kinase phosphorylates the inositol ring of PI at the D3 position (Whitman *et al.*, 1988). That PI-3′ kinase activity is regulated *in vivo* by receptor tyrosine kinases has been shown by the finding that stimulation of βPDGFR results in the rapid accumulation of PI-3,4P2 and PI-3,4,5P3 (Auger *et al.*, 1989), although the role of these novel phospholipids in cell signalling is unknown. In addition to association with activated β and αPDGFR (Kaplan *et al.*, 1987; Heidaran *et al.*, 1991), PI-3′ kinase activity associates with ligand activated forms of CSF-1R (Shurtleff *et al.*, 1990; Reedijk *et al.*, 1990) and insulin receptor (Endemann *et al.*, 1990;

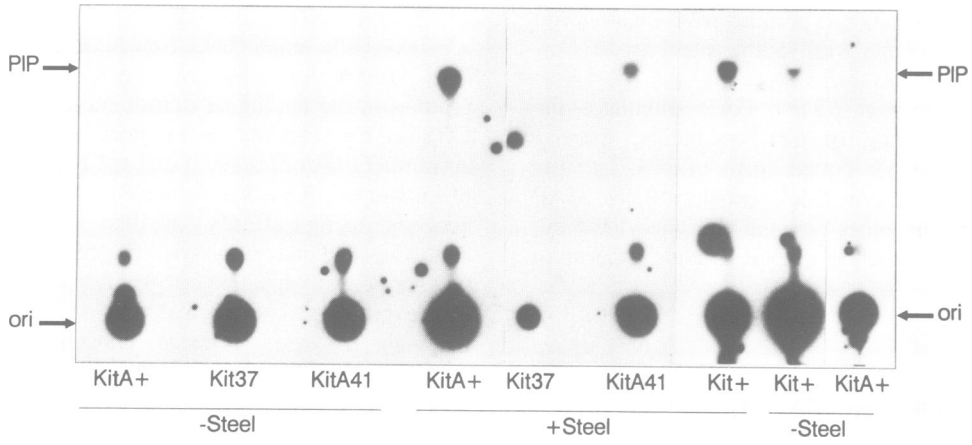

Fig. 7. Association of normal and *W* mutant Kit isoforms with PI-3′ kinase activity on Steel factor stimulation. COS cells transfected with pKit+, pKitA+, pKit37 or pKitA41 were stimulated with recombinant soluble Steel factor, where indicated, lysed and subjected to immunoprecipitation with Kit antisera. Immunoprecipitates were then assayed for associated PI-3′ kinase activity as described previously (Fukui and Hanafusa, 1989).

Ruderman *et al.*, 1990). To address a possible role for PI-3′ kinase activity in Kit signal transduction, and perturbation in *W*/c-*kit* point mutated proteins, Kit immunoprecipitates prepared from COS cell transfectants were assayed for associated PI-3′ kinase activity.

As shown in Figure 7, the KitA+ isoform associated with PI-3′ kinase activity only after stimulation with soluble Steel factor. In contrast, the Kit+ isoform was found to be associated with some PI-3′ kinase activity in the absence of exogenous ligand, and with increased amounts of PI-3′ kinase activity following stimulation with soluble Steel factor. Consistent with the deficiences in *in vitro* kinase activity and ligand mediated autophosphorylation levels, Kit37 failed to associate with any PI-3′ kinase activity, either in the presence or absence of exogenous Steel factor, whilst KitA41 exhibited a decrease in associated PI-3′ kinase activity on Steel factor stimulation (Figure 7).

Discussion

The Kit signalling pathway

The identification of Steel factor as a Kit ligand (Huang *et al.*, 1990; Williams *et al.*, 1990; Zsebo *et al.*, 1990b) has facilitated not only the biochemical definition of other components of Kit-mediated signal transuction pathways, but also analysis of the consequences of polymorphisms and point mutations in the Kit receptor on the activity of this signalling pathway. Such information complements the genetic evidence for a role of such pathways in the normal development of hematopoeitic, melanogenic and germ cell lineages in the mouse.

Steel factor stimulation results in increased tyrosine phosphorylation of Kit that is reduced or absent, respectively, in the kinase deficient W^{41} or W^{37} mutant forms of the Kit receptor, suggesting that interaction of Kit with its ligand activates the autophosphorylation activity of the intracellular Kit kinase domain. Consistent with data from other ligand activated RTKs (Kazlauskas and Cooper, 1989, 1990; Kazlauskas *et al.*, 1990; Margolis *et al.*, 1990a,b; Morrison *et al.*, 1990; Shurtleff *et al.*, 1990; Escobedo *et al.*, 1991), tyrosine phosphorylation of ligand-stimulated Kit appears to play a key role in mediating the formation of intracellular Kit signalling complexes. This function is further emphasized by the observation that increased tyrosine phosphorylation of the Kit receptor was the only change detectable by anti-phosphotyrosine Western blot analysis of whole cell lysates of Steel factor-stimulated COS cell transfectants.

When compared with the signalling complexes of other RTKs such as βPDGFR, CSF-1R and EGFR, it is apparent that the Kit receptor utilizes a unique repertoire of signalling substrates. Such distinctions in substrate association are likely to provide an important specificity to cell signalling mediated by Steel factor/Kit interactions. Association with PI-3′ kinase activity appears to be the predominant activity of ligand activated Kit. In contrast, GAP, and associated p190 and p62 proteins, are not major substrates of the activated Kit receptor. The association with PLCγ1 suggests that this protein may also be a component of the Kit signal transduction complex, a conclusion supported by the observed association of activated Kit receptors with PLCγ1 in mast cell cultures (Rotappel *et al.*, 1991). In both cases a 150 kDa tyrosine phosphorylated protein was specifically immunoprecipitated with affinity-purified PLCγ1 antisera on Steel factor stimulation of cells expressing functional Kit receptors. Whilst we have been unable to demonstrate unequivocally that this 150 kDa protein is tyrosine phosphorylated PLCγ1, EGF stimulation of an EGFR-Kit chimeric receptor has recently been shown to result in modest tyrosine phosphorylation of PLCγ1 (Lev *et al.*, 1991), although no stable association between PLCγ and the chimeric receptor was observed. Taken together, these data suggest that PLCγ1 represents a minor component of the Kit signal transduction pathway.

Kit+ and KitA+ isoforms exhibit distinct signalling properties in COS cells

The isolation of two isoforms of the Kit receptor in homogenous populations of mast cells suggest that both isoforms are co-expressed in some cell types. The presence of consensus 5′ splice junction sequences on either side of the NRI, present in KitA+ but not Kit+ proteins, indicates that such isoforms may result from differential splicing of a primary c-*kit* transcript. In this respect, it is interesting to note that the putative 5′ splice junction involved in the formation of Kit+ and KitA+ isoforms is the same as that

proposed to be utilized in the aberrant RNA splicing associated with the original *W* mutation (Nocka *et al.*, 1990a). Confirmation of such differential splicing models awaits the molecular definition of the relevant region of the c-*kit* locus.

Whilst both Kit isoforms were activated by soluble Steel factor, and associated with the same subset of substrates, the inclusion of NRI in the KitA+ isoform abolished a low constitutive level of Kit signal transduction mediated by the Kit+ isoform in the absence of an exogenous source of Kit ligand. The mechanism underlying this observation is presently unclear. High levels of protein expression achieved in COS cells may result in spontaneous dimerization of receptors in the absence of ligand, in which case NRI would confer a lower efficiency of receptor aggregation. It is also possible that the inclusion of NRI in the extracellular domain confers a differential response to ligands present in serum or produced by COS cells. The variation in the constitutive tyrosine phosphorylation of Kit+ as a function of COS cell culture conditions is consistent with this view. Since COS cells do not support murine mast cell growth, any constitutive signalling mediated by COS cells on the Kit+ isoform is presumably below the threshold required to evoke a mitogenic response in mast cells, and would be further reduced in mast cells since they express both COS cell-responsive and unresponsive Kit isoforms. The demonstration of distinct signalling properties of normal Kit isoforms co-expressed in mast cells and placenta and conserved between mouse and man, raises the possibility that they may play important roles in mediating appropriate diversity and regulation in the response of Kit signalling pathways to specific changes in cellular microenvironments during mammalian development.

W mutant phenotypes and RTK-mediated signalling pathways in development

W mutant phenotypes have been attributed to loss of function mutations in the Kit receptor tyrosine kinase. Of 11 independent *W* alleles anlaysed to date, all bear either regulatory or structural mutations that confer a partial or complete loss of Kit *in vitro* kinase activity (Chabot *et al*, 1988; Geissler *et al.*, 1988; Bernstein *et al.*, 1990; Nocka *et al.*, 1989, 1990a; Dubreuil *et al.*, 1990; Reith *et al.*, 1990; Tan *et al.*, 1990). A complete loss of Kit *in vitro* kinase activity is associated with homozygous lethal phenotypes, whilst residual levels of Kit *in vitro* kinase activity are compatible with viability, although such animals bear defects of varying severity in development of hematopoietic, germ cell and melanogenic lineages (Dubreuil *et al.*, 1990; Reith *et al.*, 1990).

With the exception of the Asp790 → Asn mutation identified in the W^{42} allele and previously mutated in the v-*fps* oncogene (Moran *et al.*, 1988; Tan *et al.*, 1990), none of the single amino acid substitutions associated with structural *W* mutant alleles have been previously shown to be necessary for normal activity of tyrosine kinases. Here, we have provided direct biochemical evidence that the Glu582 → Lys and Val831 → Met point mutations previously identified in c-*kit* cDNA from W^{37} and W^{41} mutant mice, respectively, confer deficiencies in Kit kinase activity and in the ability of Kit to be activated by its ligand and associated with PI-3′ kinase activity and PLCγ1. Thus, the homozygous lethal and homozygous viable W^{37} and W^{41} mutant phenotypes can now be understood in molecular terms as resulting from the complete or partial inability of Kit to assemble an intracellular signal transduction complex in response to growth factor stimulation, a direct consequence of the Glu582 → Lys and Val831 → Met primary mutations in the Kit receptor tyrosine kinase.

The W^{41} allele has distinct effects on different cell lineages, W^{41}/W^{41} animals being almost all white, having a relatively severe macrocytic anemia, but retaining normal fertility (Geissler *et al.*, 1981). In this respect, it is interesting to note that only quantitative reductions in substrate association were observed for the W^{41} mutated form of Kit. Thus, it is possible that the residual level of Kit signalling conferred by the W^{41} allele is sufficient for normal germ cell development, but not normal melanogenesis or hematopoiesis. Alternatively, other signalling pathways, with components overlapping those of the Steel factor/Kit pathway, may supplement the residual level of W^{41} Kit signalling in germ cells in a manner analogous to that shown for the c-*fms* mediated complementation of *W* mutant mast cells in a fibroblast co-culture growth assay (Dubreuil *et al.*, 1991). The utilization of overlapping subsets of intracellular substrates by Kit and other RTKs may also account for the absence of detectable *W* or *Sl* mutant defects in tissues such as the nervous system and kidney, where co-expression of Steel factor and c-*kit* have been shown by RNA *in situ* hybridization analyses (Matsui *et al.*, 1990; Orr-Urtreger *et al.*, 1990; Motro,B., van der Kooy,D., Rossant,J., Reith, A.D., Williams,D.E., Lyman,S.D., Anderson,D.M. and Bernstein,A., submitted for publication).

A corollary of this is that loss of function germline mutations in PI-3′ kinase, PLCγ1, or other, as yet unidentified, downstream components of the Kit signalling pathway, may give rise to animals with mutant phenotypes overlapping those of *W* or *Sl* mutants. Many existing mutant strains of mice bear defects in melanogenesis, hematopoiesis or germ cell development (Lyon and Searle, 1989) and molecular analysis of one mutant locus in this class, *microphthalmia (mi)* (Hertwig, 1942), has already provided evidence that it may encode some component(s) downstream of, and common to, Kit and Fms signalling pathways (Dubreuil *et al.*, 1991). Together with the generation of novel germline mutations in known components of signal transduction pathways, further molecular analyses of existing mouse mutant stocks will undoubtedly provide greater insight into the functions of RTK-mediated signalling pathways in mammalian development.

Materials and methods

Nucleic acids

The murine c-*kit* expression plasmid pKitA+ was constructed by insertion of a 3.7 kb murine c-*kit* cDNA clone (Reith *et al.*, 1990) into the SV40-based mammalian expression vector pECE (Ellis *et al.*, 1986). Nucleotide sequence analysis showed that pKitA+ contains the 12 nucleotide asparagine-rich insert (NRI) between amino acids 512 and 513 in the published murine c-*kit* cDNA sequence (Qui *et al.*, 1988; Figure 1). pKit+, lacking NRI, was derived from pKitA+ by substitution of the *Nde*I−*Pfl*M1 fragment of pKitA+ with that from the PCR generated cDNA clone W55-11, the nucleotide sequence of which was determined to be identical to that previously published (Reith *et al.*, 1990). pKit37, was derived from pKitA+ by substitution of the *Nde*I−*Pfl*M1 fragment of pKitA+ with that from the PCR generated c-*kit* cDNA clone W37-2-52 that lacks NRI but bears the G/C → A/T point mutation characteristic of the W^{37} allele (Reith *et al.*, 1990). pKitA41 was derived from pKitA+ by substitution of the *Nhe*I−*Aat*II restriction fragment with that from the PCR generated c-*kit* cDNA clone

W41-1-4A4 the nucleotide sequence of which bears NRI was well as the single base substitution characteristic of the W^{41} mutant c-*kit* allele (Reith *et al.*, 1990). PCR cDNA cloning and nucleotide sequence analysis was as described previously (Reith *et al.*, 1990). pmtPDGFR contains a human βPDGFR cDNA subcloned into the pmt vector (J.Knopf, personal communication). pECEv-src has been described previously (Brooks-Wilson *et al.*, 1989). Preparation of nucleic acids for transfection was performed by standard methods.

Cell culture

The SV40-transformed monkey kidney cell line COS-1 (Gluzman, 1981) was routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Flow) grown in an incubator at 37°C gassed with 5% CO_2, 100% relative humidity. For DNA transfections, cells were seeded at 4×10^5 per 60 mM plate and the following day transfected with 5 μg DNA using 25 μl lipofectin reagent (BRL) in 3 ml OptiMEM (Gibco) for 5 h. An equal volume of DMEM, 20% FCS was added and cells incubated overnight prior to being refed with DMEM, 10% FCS. 8–32 h later, as appropriate, cells were refed with DMEM, 0.5% FCS and incubated for a further 16–40 h prior to being assayed.

For metabolic labelling, cells were cultured for 4 h in methionine-free DMEM (Gibco) supplemented with 5% dialysed FCS (Flow) and 100 μCi/ml [^{35}S]methionine (Amersham). For growth factor stimulation experiments, 1 ml medium was removed per plate, pooled, supplemented with 1 μg/ml yeast recombinant murine soluble Steel factor (Immunex Corp.), 80 nM, epidermal growth factor (UBI), or 75 nM platelet-derived growth factor (BB homodimer) (UBI), as appropriate, and warmed to 37°C. The remaining medium was removed from each plate, 1 ml prewarmed medium +/− growth factor added, and cells incubated at 37°C for 5 min.

Immunoprecipitation analyses

Immunoprecipitation and SDS–PAGE analysis of [^{35}S]methionine-labelled cells were performed as described previously (Reith *et al.*, 1990).

For whole cell lysate assays, a 60 mm plate of confluent cells was lysed in 200 μl hot SDS sample buffer. The lysate was heated for 3 min at 100°C, forced five times through a 25 gauge needle, assayed to determine protein concentration, and equivalent masses of protein subjected to SDS–PAGE. For immunoprecipitation of Steel factor stimulated cultures, cells were placed on ice, washed twice with ice cold Tris–saline and lysed in 1 ml PLC lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, 200 μM sodium orthovanadate, 10 mM sodium fluoride). Extracts were precleared in an Eppendorf microfuge at 4°C for 15 min and the supernatant then subjected to immunoprecipitation for 90 min at 4°C, using anti-kit rabbit polyclonal antisera (Reith *et al.*, 1990), anti-PLCγ (Decker *et al.*, 1990), or anti-GAP (Ellis *et al.*, 1990) rabbit polyclonal antibodies, or affinity-purified rabbit anti-phosphotyrosine antibodies (Letwin *et al.*, 1988) and 100 μl 10% protein A–Sepharose. Immune complexes were washed three times with HNTG (20 mM HEPES, pH 7.5, 10% glycerol, 0.1% Triton X-100, 150 mM NaCl and 1 mM sodium orthovanadate).

In vitro kinase assays

Immunoprecipitations, *in vitro* kinase assays and SDS–PAGE were performed as described previously (Reith *et al.*, 1990).

Western blots

Immune complexes were heated for 3 min at 100°C in SDS sample buffer. Immunoprecipitated proteins were separated by 7.5% SDS–PAGE, transferred to nitrocellulose, and probed with affinity purified anti-phosphotyrosine antibodies, or anti-PLC-γ antibodies as described previously (Ellis *et al.*, 1990).

PI 3′-kinase assays

Anti-kit immunoprecipitates, prepared as described above, were washed and assayed for *in vitro* PI-3′ kinase activity, as described by Fukui and Hanafusa (1989).

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Note added in proof

Hayashi *et al.* (*Nucleic Acids Res.*, **19**, 1267–1271) have recently reported the genomic structure of the c-*kit* locus in the region of the differentially spliced NRI.