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# Phosphorylation of Shc by Src family kinases is necessary for stem cell factor receptor/c-*kit* mediated activation of the Ras/MAP kinase pathway and c-*fos* induction

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**In this report we show that Tyr568 and Tyr570 are phosphorylated *in vivo* in the Kit/stem cell factor receptor (Kit/SCFR) following ligand-stimulation. By mutation of Tyr568 and Tyr570 to phenylalanine residues and expression of the mutated receptors in porcine aortic endothelial (PAE) cells, we could demonstrate a loss of activation of members of the Src family of tyrosine kinases when Tyr568 was mutated, while mutation of Tyr570 only led to a minor decrease in activation of Src family members. Mutation of both tyrosine residues led to a complete loss of Src family kinase activation. Phosphorylation of the adapter protein Shc by growth factor receptors provides association sites for Grb2-Sos, thereby activating the Ras/MAP kinase pathway. A much lowered degree of Shc phosphorylation, Ras and Erk2 activation and c-*fos* induction was seen in the Y568F mutant, while in the Y570F mutant these responses were less affected. In contrast, the mitogenic response was only slightly reduced. In a mutant receptor with both Tyr568 and Tyr570 mutated to phenylalanine residues, no phosphorylation of Shc and no activation of Ras and Erk2 was seen in response to stem cell factor stimulation, very weak induction of c-*fos* was seen and the mitogenic response was severely depressed. These data show that Ras/MAP kinase activation and c-*fos* induction by Kit/SCFR are mediated by members of the Src family kinases. However, the mitogenic response is only to a minor extent dependent on Src kinase activity.**

**Keywords:** c-*kit*; Src; Ras; MAP kinase; Shc; mitogenicity

## Introduction

The c-*kit* proto-oncogene encodes a receptor protein tyrosine kinase, which is structurally related to the receptors for platelet-derived growth factor (PDGF)[1], colony stimulating factor-1 (CSF-1) (Yarden *et al*., 1987) and Flk-2/Flt-3. The c-*kit* gene is allelic with the murine *dominant white spotting (W)* locus, while its ligand, stem cell factor (SCF), is encoded by the murine *steel (Sl)* locus (Witte, 1990). A number of mutations at these loci have been described both in mice and humans, and give rise to defects in migration, differentiation and proliferation of cells in the melanogenic, gametogenic and hematopoietic lineages (for reviews, see Besmer, 1991 and Fleischman, 1993).

Binding of SCF to the Kit/SCF receptor (Kit/SCFR) induces dimerization and autophosphorylation of the receptor on tyrosine residues (Blume-Jensen *et al*., 1991; Herbst *et al*., 1991; Lev *et al*., 1991), thereby creating docking sites for cytoplasmic signaling molecules containing Src homology 2 (SH2) domains (Pawson, 1995). SH2 domains are found in many signal transduction molecules and consist of a stretch of about 100 amino acids which bind phosphorylated tyrosine residues in a specific amino acid sequence context. Several signal transduction molecules have been found to be phosphorylated by and in some cases bind to the activated Kit/SCFR, including PI3′-kinase, Vav, Grb2 and Shc (Alai *et al*., 1992; Cutler *et al*., 1993; Lev *et al*., 1991; Tauchi *et al*., 1994). Several activating mutations of Kit/SCFR have been found in mast cell leukemias and gastrointestinal stroma tumors (Furitsu *et al*., 1993; Hirota *et al*., 1998). Interestingly, many of these mutations are either point mutations or deletion mutations in the juxtamembrane region of the Kit/SCFR. Furthermore, the retroviral oncogene v-*kit* encodes a protein containing deletions in the juxtamembrane region (Herbst *et al*., 1995). This prompted us to investigate the function of the juxtamembrane region in Kit/SCFR. Here we demonstrate that Tyr568 and Tyr570, located in the juxtamembrane domain, are phosphorylated *in vivo* following SCF-stimulation. We could demonstrate that binding and activation of the Src family kinases is dependent of these phosphorylation sites. Furthermore, phosphorylation of Shc, activation of Ras and Erk2 and induction of c-*fos* by SCF is dependent on phosphorylation of Tyr568. Thus, it is likely that the Kit/SCFR activated Src kinases are responsible for phosphorylation of Shc, leading to activation of the Ras/MAP kinase pathway.

## Results

### *Identification of Tyr568 and Tyr570 as* in vivo *autophosphorylation sites in the Kit/SCFR*

In order to investigate the potential presence of autophosphorylated tyrosine residues in the juxtamembrane region of Kit/SCFR, cells were labeled with $^{32}$P-orthophosphate, stimulated with SCF, lysed and

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immunoprecipitated with Kit/SCFR specific antibodies. The immunoprecipitated protein was resolved on a SDS-polyacrylamide gel, transferred to Hybond C-extra membrane and digested *in situ* with trypsin. The tryptic peptides were subjected to immunoprecipitation using a peptide antiserum, VVE, raised against a synthetic peptide corresponding to the juxtamembrane domain. After washing, the immunoprecipitated peptide was eluted and coupled covalently to a Sequelon AA membrane and subjected to automated Edman degradation. Radioactivity released in each cycle of Edman degradation was quantitated. An aliquot of the immunoprecipitated peptide was also subjected to phosphoamino acid analysis. Two peaks of radioactivity were seen, in cycles 10 and 12, corresponding to Tyr568 and Tyr570 (Figure 1). In contrast, no radioactivity could be detected in cycle 20, corresponding to Tyr578. Furthermore, upon performing a similar experiment on PAE cells expressing the Y568F or Y570 mutant Kit/SCFR, no peak of radioactivity was seen in cycle 10 or 12, respectively, upon sequencing of the tryptic juxtamembrane peptide (data not shown). In the Y568F/Y570F double mutant cell line, no radioactivity was brought down in the peptide comprising the juxtamembrane region, further supporting the notion that Tyr578 is not phosphorylated in the Kit/SCFR (data not shown).

*The role of Tyr568 and Tyr570 in binding and activation of members of the Src family of protein tyrosine kinases*

Tyr568 and Tyr570 in Kit/SCFR correspond to Tyr579 and Tyr581 in the PDGF $\beta$-receptor and Tyr572 and Tyr574 in the PDGF $\alpha$-receptor (Gelderloos *et al*., 1998; Hooshmand-Rad *et al*., 1998; Mori *et al*., 1993). This prompted us to test whether Tyr568 and Tyr570 are necessary for binding and activation of Src family members by Kit/SCFR.

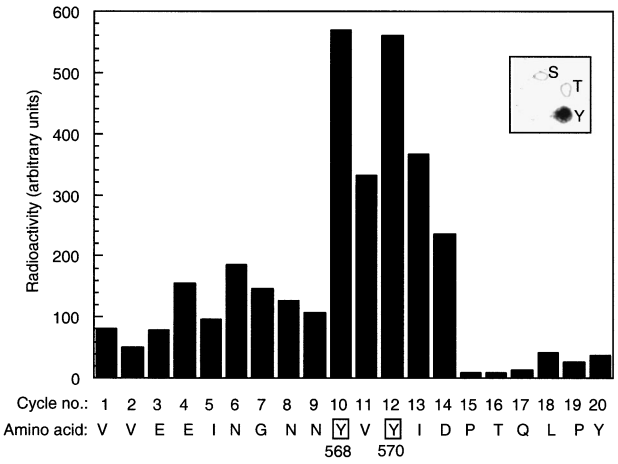



**Figure 1** Identification of Tyr568 and Tyr570 as autophosphorylation sites in the Kit/SCFR. PAE/kit cells were incubated with [$^{32}$P]orthophosphate, stimulated with SCF, lysed and the Kit/SCFR immunoprecipitated. Proteins were separated by SDS–PAGE, electrotransferred to nitrocellulose and digested *in situ* with trypsin. Tryptic fragments were subjected to immunoprecipitation using the VVE antiserum, washed and eluted with 1% diethylamin. Immunoprecipitated peptides were subjected to Edman degradation. The radioactivity released in each cycle of Edman degradation was quantitated and plotted. An aliquot of phosphopeptide was subjected to phosphoamino acid analysis

We therefore mutated Tyr568 and Tyr570 to phenylalanine residues, either individually or in combination, and expressed the mutated Kit/SCFR in PAE cells. The mutant cell lines expresed mutant Kit/SCFR to a similar level as wild-type Kit/SCFR expressing PAE cells. Stimulation of PAE cells expressing wild-type Kit/SCFR with SCF led to a strong induction of enolase kinase activity in cst-1 immunoprecipitates. The cst-1 antiserum recognizes c-Src, c-Fyn and c-Yes of the Src family of tyrosine kinases, which previously have been shown to be the major Src kinases in PAE cells. In contrast, cells expressing the Y568F mutant showed a complete loss of Src kinase activation, while the Y570F mutant showed only a minor decrease in Src kinase activity.

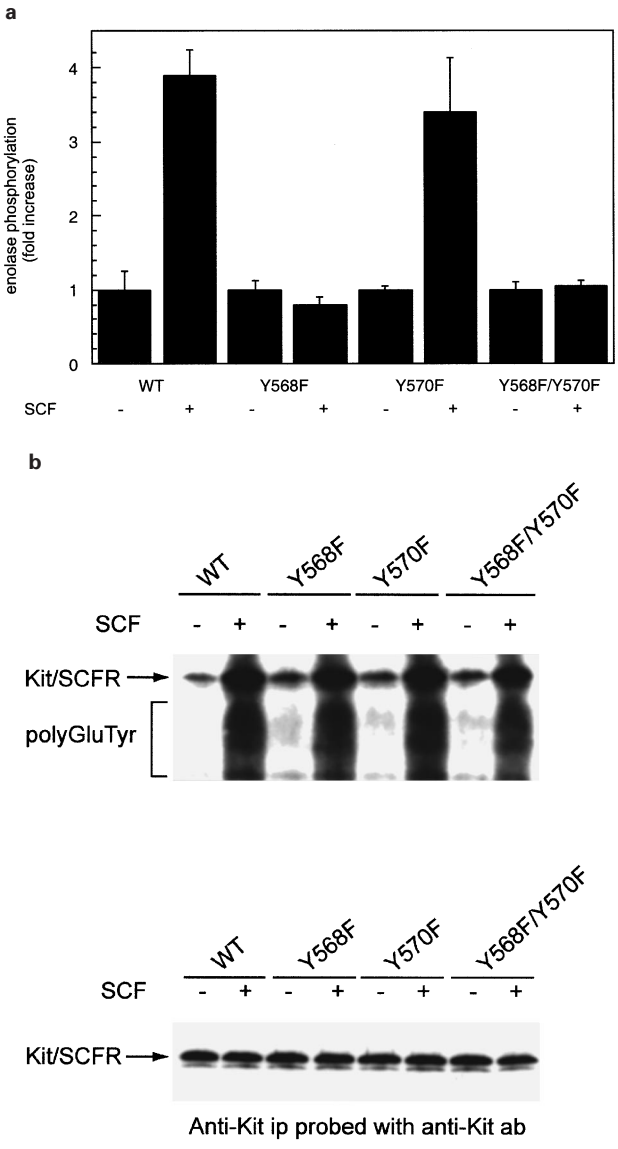



**Figure 2** SCF-induced activation of Src family kinases is primarily dependent on Tyr568. PAE cells expressing either wild-type Kit/SCFR, Y568F mutant, Y568F mutant or Y568F/Y570F double mutant Kit/SCFR were stimulated with SCF, lysed and subjected to immunoprecipitation using either the cst-1 antiserum, recognizing c-Src, c-Yes and c-Fyn (**a**) or KitC1 antibodies, recognizing Kit/SCFR (**b**). The washed immunoprecipitates were incubated with $\gamma$[$^{32}$P]ATP and acid-denatured enolase (**a**) or polyGluTyr (**b**) for 30 min. Proteins were separated by SDS–PAGE and exposed either on X-ray film or on a Fuji BAS2000 Image Analyzer. The amount of radioactivity incorporated into enolase was quantitated

The double mutant was devoid of SCF-inducible Src kinase activity (Figure 2a).

To rule out the possibility that the loss of Src kinase activation was due to a general loss of Kit/SCFR kinase activity, cells expressing wild-type Kit/SCR, Y568F mutant, Y570F mutant or Y568F/Y570F mutant Kit/SCFR were stimulated with SCF, lysed and immunoprecipitated using an anti-Kit/SCFR antibody. The washed immunoprecipitates were subjected to an *in vitro* kinase assay using poly-GluTyr (4 : 1) as an exogenous substrate. It could be clearly demonstrated that the different mutants exhibited a kinase activity of the same magnitude as the wild-type Kit/SCFR (Figure 2b).

To investigate whether Src family kinases can associate with Kit/SCFR, cells expressing Kit/SCFR were stimulated with SCF, lysed, immunoprecipitated with cst-1 antibodies; immunoprecipitates were then separated by SDS-gel electrophoresis and subjected to immunoblotting with anti-phosphotyrosine antibodies. A weak tyrosine phosphorylated band was seen to co-immunoprecipitate with Src kinases. However, immunoblotting with Kit/SCFR specific antibodies failed to detect this band (data not shown).

A more sensitive assay for association with Kit/SCFR is the use of GST-fusion proteins containing the SH2 domain(s) of signal transduction molecules. Incubation of Triton-lysate from SCF-stimulated Kit/SCFR expressing cells with GST-SrcSH2, led to association of Kit/SCFR with the fusion protein (Figure 3). To assess the specificity of the interaction, a competition experiment was performed using synthetic peptides corresponding to the amino acid sequence surrounding Tyr568 and Tyr570, either phosphorylated at Tyr568, Tyr570, or both, or unphosphorylated (pY568, pY570, pY568/pY570 and Ref, respectively). As can be seen (Figure 3), the p568 peptide strongly competed binding of the Kit/SCFR to the GST-SrcSH2 fusion protein, the p570 peptide competed weakly, while the p568/p570 competed strongest with binding of the Kit/SCFR to the Src SH2 domain. These results are in agreement with the data on Src kinase activity, i.e. Tyr568 is the primary site of association, while Tyr570 contributes to the full binding.

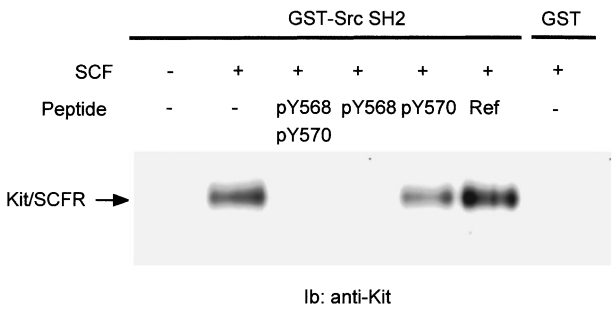



**Figure 3** Association of the Kit/SCFR with the SH2 domain of c-Src and its competition with phosphorylated peptides containing Tyr568 and Tyr570. PAE/kit cells were stimulated with SCF, lysed and incubated with GST-SrcSH2 immobilized to glutathione-Sepharose, in the presence or absence of peptides pY568, pY570, pY568/pY570 or non-phosphorylated reference peptide (Ref) (see Materials and methods). Bound Kit/SCFR was detected by Western blotting using the KitC1 antibody

*Mutation of Tyr568 and Tyr570 to phenylalanine residues leads to loss of ligand-stimulated Ras GTP-loading and MAP kinase activation*

The Ras/MAP kinase pathway, that has been demonstrated to be of utmost importance for mitogenic signaling in a number of growth factor systems, is activated by the Kit/SCFR following ligand-stimulation (Blume-Jensen *et al*., 1995; Funasaka *et al*., 1992). In order to investigate the role of Tyr568 and Tyr570 in Kit/SCFR mediated Ras/MAP kinase signaling, PAE cells expressing either wild-type, Y568F mutant, Y570F mutant or the Y568F/Y570F double mutant Kit/SCFR were challenged with SCF and the time course of Erk2 activation was measured by an *in vitro* kinase assay using myelin basic protein as a substrate. We could demonstrate that the SCF-induced activation of Erk2 was depressed in the Y570F mutant cell line, and almost completely abolished in the Y568F mutant cell line. In the Y568F/Y570F double mutant cell line no activation of Erk2 was seen (Figure 4). Since Ras activation is the first step in the MAP kinase cascade, we further investigated the influence of the Y568F and Y570F mutations on SCF-stimulated Ras-GTP loading. Ras GTP-loading increased rapidly following stimulation with SCF, peaking at 5 min, and then slowly leveled off. As with Erk2 activation, the SCF-induced increase in Ras GTP-loading was severely depressed in the Y568F and Y570F single mutant expressing cells, while no increase in Ras GTP-loading was seen in the Y568F/Y570F double mutant cells (Figure 5). As a control, FCS-stimulated Ras GTP-loading and Erk2 kinase activation was seen to a similar extent in all cell lines tested (data not shown).

*Phosphorylation of Shc is dependent on Tyr568 and Tyr570*

The adapter protein Shc has previously been shown to be phosphorylated by and/or associate with a number

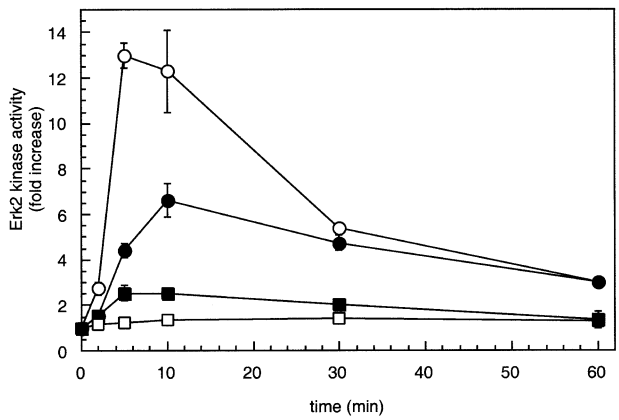



**Figure 4** SCF-stimulated Erk2 kinase activity correlates with Src activation. PAE cells expressing either the wild-type Kit/SCFR (○), Y568F mutant (■), Y570F mutant (●), or Y568F/Y570F mutant (□) receptors were stimulated with SCF for the indicated time, lysed and immunoprecipitated with an anti-Erk2 antiserum. Washed immunoprecipitates were incubated with $\gamma[^{32}P]$ATP and myelin basic protein. Samples were separated on SDS–PAGE and the amount or radioactivity incorporated in myelin basic protein quantitated

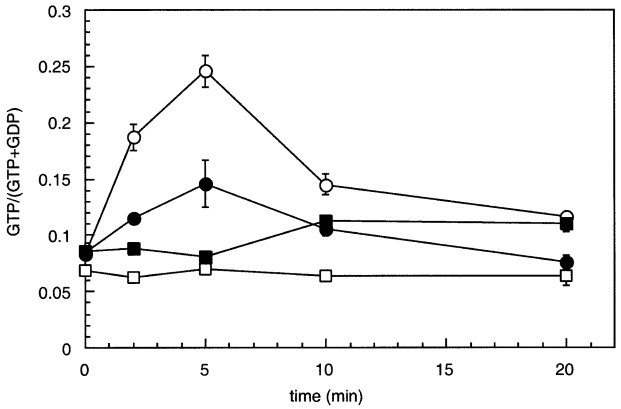
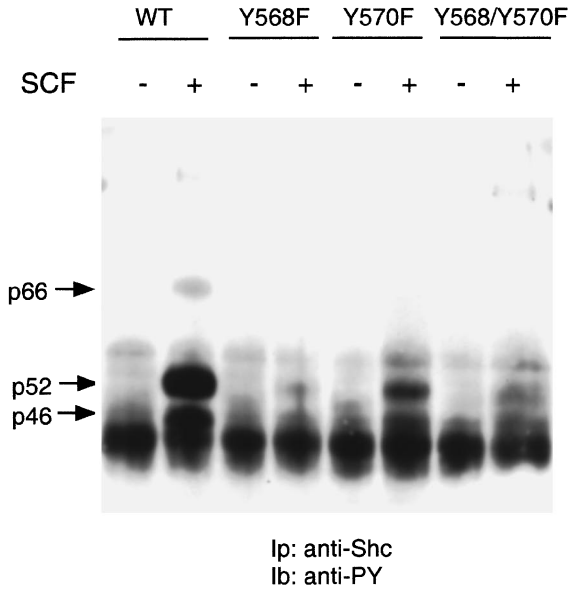



**Figure 5** The rapid SCF-stimulated increase in Ras GTP loading is dependent on Tyr568. PAE cells expressing either the wild-type Kit/SCFR (○), Y568F mutant (■), Y570F mutant (●) or Y568F/Y570F mutant (□) receptors, were stimulated with SCF for the indicated time, lysed and immunoprecipitated with a Ras antibody, Y13-259. The immunoprecipitates were extensively washed. Bound GTP and GDP was resolved on PEI-cellulose plates and quantitated

of growth factor receptors (Cutler *et al*., 1993; Sasaoka *et al*., 1994; Yokote *et al*., 1994). We have previously shown that the SH2 domain of Shc does not associate with the autophosphorylated Kit/SCFR (Blume-Jensen *et al*., 1994). However, when assaying for tyrosine phosphorylation following SCF stimulation of PAE/kit cells, a strong phosphorylation was seen of both the p46 and p52 forms of Shc (Figure 6). A weak phosphorylation of the p66 isoform was also seen. In contrast, phosphorylation of Shc was strongly reduced in PAE cells expressing the Y568F mutant Kit/SCFR. In cells expressing the Y570F mutant, phosphorylation of Shc was of intermediate intensity. Furthermore, in the Y568F/Y570F mutant cell line almost no SCF-induced tyrosine phosphorylation of Shc was seen (Figure 6). Thus, phosphorylation of Shc correlates with activation of Src family kinases.

### *Phosphorylation of Tyr568 and Tyr570 is necessary for induction of c-*fos *by the Kit/SCFR*

It is well established that growth factor stimulated c-*fos* induction is dependent on the Ras/MAP kinase pathway (Gille *et al*., 1992). MAP kinases have been demonstrated to phosphorylate Elk-1, leading to enhanced formation of the ternary complex and induction of c-*fos* Gille *et al*., 1995). Furthermore, it has recently been shown that MAP kinase dependent activation of the serine/threonine kinase Rsk-2 can mediate c-*fos* induction (De Cesare *et al*., 1998). Phosphorylation of Shc at Tyr317 has been demonstrated to mediate Ras/MAP kinase activation and c-*fos* induction. PAE cells expressing either wild-type Kit/SCFR, Y568F mutant, Y570F mutant or Y568F/Y570F mutant Kit/SCFR were stimulated with SCF, RNA was prepared and Northern blotting performed using a probe against c-*fos*. It could be seen that induction of c-*fos* was strongly induced by SCF in wild-type Kit/SCFR expressing PAE cells, with a peak in induction at 30 min after stimulation. In contrast, in the Y570F mutant c-*fos* induction was severely diminished, while in the Y568F mutant and in the



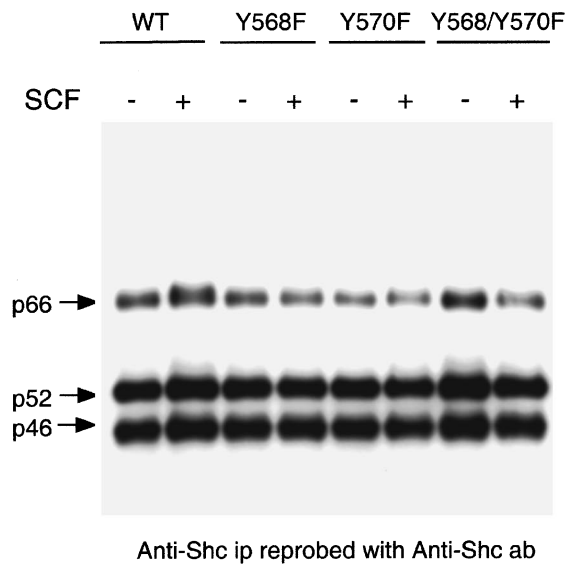




**Figure 6** SCF-induced Shc phosphorylation is dependent on Src activation. PAE cell expressing either the wild-type Kit/SCFR, Y568F mutant Y570F mutant or Y568F/Y570F mutant receptors, were stimulated with SCF, lysed and immunoprecipitated with a Shc antiserum. Immunoprecipitates were washed, and bound proteins separated in SDS–PAGE, electrotransferred to Immobilon P and probed with an anti-phosphotyrosine antibody. The blot was then stripped and reprobed with anti-Shc antibody to demonstrate equal loading in the different lanes

Y568F/Y570F double mutant the induction of c-*fos* was even weaker (Figure 7).

### *Mutation of Tyr568 and Tyr570 in the Kit/SCFR to phenylalanine residues leads to a loss of mitogenic response to SCF*

It has previously been claimed that activation of Src family kinases is essential for Kit/SCFR mediated mitogenesis (Linnekin *et al*., 1997). Timokhina *et al*. (1998) could demonstrate decreased SCF-stimulated DNA-synthesis in cells expressing the Y568F mutant.

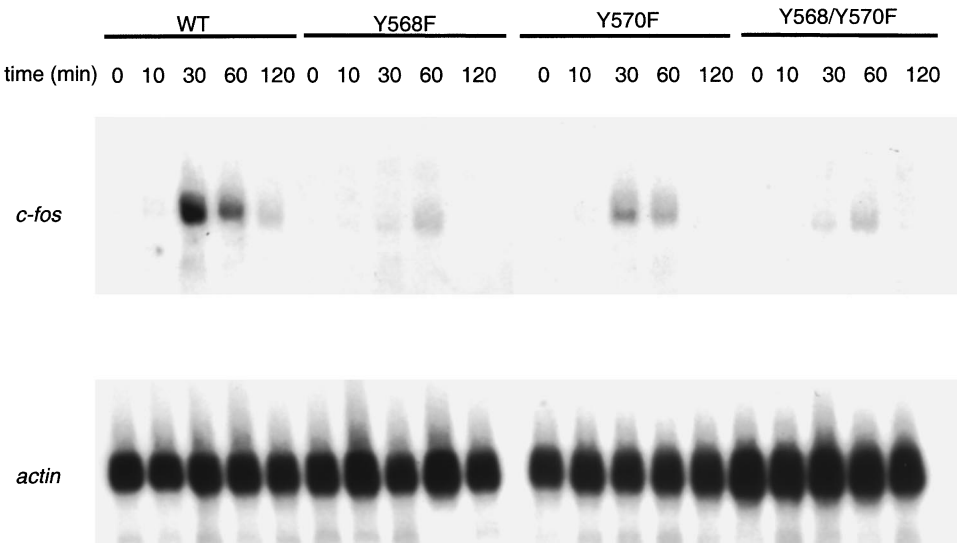



**Figure 7** Induction of c-*fos* expression in various mutant Kit/SCFR cell lines. PAE cells expressing either the wild-type Kit/SCFR, Y568F mutant, Y570F mutant or Y568F/Y570F mutant receptors, were stimulated with SCF for the indicated time periods. Cells were then lysed and RNA prepared. After separation of RNA on agarose gel and blotting to nitrocellulose filter, Northern blotting was performed using a c-*fos* probe. After stripping, the filter was incubated with an actin specific probe, to demonstrate equal loading in each lane

In contrast, Kozlowski *et al*. (1998) showed an increased mitogenicity in cells expressing either the Y568F or the Y570F mutant Kit/SCFR (Kozlowski *et al*. 1998).

We could show that PAE cells expressing either the Y568F or the Y570F mutant Kit/SCFR showed a slight decrease in SCF-induced DNA-synthesis, while the Y568F/Y570F double mutant expressing PAE cells showed a dramatic reduction in SCF-induced DNA-synthesis (Figure 8). Thus, since there is not correlation between the ability of the different mutants to induce Src kinase activation and mitogenicity, it is likely that pathways other than Src kinase activation contribute to Kit/SCFR mediated mitogenic signaling.

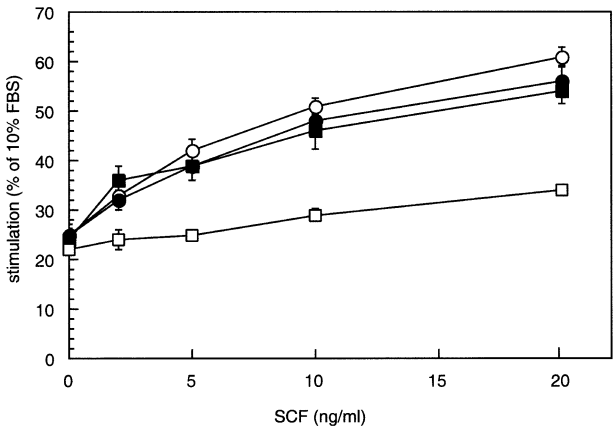



**Figure 8** SCF-induced mitogenicity is severely depressed only if both Tyr568 and Tyr570 are mutated. PAE cells expressing either wild-type Kit/SCFR (○), Y568F mutant (■), Y570F mutant (●) or Y568F/Y570F double mutant (□) Kit/SCFR were starved overnight in 1% BSA. Cells were then stimulated with the indicated concentration of SCF for 24 h in the presence of [$^3$H]thymidine. The amount of [$^3$H]thymidine incorporated into DNA was determined using a scintillation counter

## Discussion

The juxtamembrane region of Kit/SCFR is of great regulatory importance. Numerous naturally occurring activating mutations found in Kit/SCFR reside in the juxtamembrane region, either as point mutations or deletions (Furitsu *et al*., 1993; Hirota *et al*., 1998). Furthermore, the viral form of Kit/SCFR, v-*kit*, contains deletions in its juxtamembrane region that accounts for its tumorigenic potential (Herbst *et al*., 1995). Five tyrosine residues are located in the juxtamembrane region, out of which two are phosphorylated *in vivo* following SCF-stimulation. Tyr568 and Tyr570 have been shown by several groups to be phosphorylation sites (Kozlowski *et al*., 1998; Linnekin *et al*., 1997; Price *et al*., 1997; Timokhina *et al*., 1998). Linnekin *et al*. (1997) could show that these tyrosine residues mediate binding of the Src family member Lyn in Mo7e cells, and demonstrate by an antisense approach and by the use of the Src inhibitor PP1 the importance of Src family kinases for Kit/SCFR mediated mitogenesis. However, the significance of these data is unclear, since the inhibitor used, PP1, is also a potent inhibitor of Kit/SCFR kinase activity at the concentrations used (Lennartsson *et al*., unpublished observation).

Decreased Ras/MAP kinase activation and decreased c-*fos* induction was seen in the Y568F and Y568F/Y570F mutant PAE cell lines, while the Y570F mutant cell line was less affected. Previous studies on the endothelin-1 signaling, have implicated Src as a necessary component for signaling c-*fos* induction (Simonson *et al*., 1996). In the PDGF $\alpha$- and $\beta$-receptors, that are closely related to the Kit/SCFR, Src family kinases bind to the homologous Tyr572 and Tyr574 (Gelderloos *et al*., 1998; Hooshmand-Rad *et al*., 1998), and Tyr579 and Tyr581 (Mori *et al*., 1993), respectively. Src family members have been claimed to be mediators of mitogenicity and c-*myc* induction by PDGF (Barone and Courtneidge, 1995; Roche *et al*.,

1995). On the other hand, Gelderloos *et al*. (1998) saw no effect on either c-*myc* or c-*fos* induction in cells expressing the Y572F/Y574F mutant of the PDGF α-receptor, that lacks the ability to associate with and activate Src family members. The apparent discrepancy in results could in part be explained by the different techniques used. Microinjection of SH2 domain fusion proteins, will inhibit Src binding to effector molecules, but also interfere with binding of other SH2 domain containing proteins that have similar sequence specificity as Src. Microinjected molecules, dominant negative proteins or antibodies, will be able to interact with Src independent of whether it is activated at the receptor level or later in the cell cycle. On the other hand, point mutated receptors will only interfere with the binding of Src to the receptor (and possibly other molecules binding to the same phosphorylated tyrosine residues). One cannot exclude the possibility that hitherto unidentified molecule(s) bind to Tyr568 and Tyr570 in the Kit/SCFR and that this/these protein(s) in part mediate the effects observed.

Shc has previously been shown to be phosphorylated following activation of a number of growth factor receptors, at Tyr317, Tyr239 and Tyr240 (Salcini *et al*., 1994; van der Geer *et al*., 1996). Phosphorylated Tyr317 constitutes a consensus binding site for Grb2 (Salcini *et al*., 1994). Thus, Shc has been shown to be acting on one of several pathways leading from receptor tyrosine kinases to the Ras/MAP kinase pathway. In this report we show that phosphorylation of Shc following SCF stimulation of Kit/SCFR expressing cells most likely occurs through Src family kinases, not through the receptor itself. Thus, Src phosphorylation of Shc seems to constitute a pathway for Kit/SCFR mediated activation of the Ras/MAP kinase pathway, leading to, among other things, induction of c-*fos* expression.

The differences in SCF-induced DNA synthesis seen in the different mutants does not correlate with Src activation. In the Y568F and Y570F single mutants only a marginal decrease in SCF-induced DNA synthesis was seen (Figure 9), while in the Y568F/Y570F double mutant a dramatic decrease in Src activation was seen (Figure 2a). This suggests that there might be additional signal transduction molecules binding to Tyr568 and Tyr570. Using Ba/F3 cells, Kozlowski *et al*. (1998) demonstrated binding of the protein tyrosine phosphatases SHP-1 and SHP-2 to Tyr568 and Tyr570, respectively. However, in PAE cells expressing the Kit/SCFR, we were unable to detect any such interactions. Furthermore, in contrast to our findings and findings by Timokhina *et al*. (1998), Kozlowski *et al*. (1998) demonstrated an increased mitogenic response in cells expressing the Y568F and Y570F Kit/SCFR mutants. These differences in cellular response might be due to the different repertoire of signal transduction molecules expressed in the different cell types, e.g. SHP-1 is primarily expressed in hematopoietic cells, while its expression in PAE cells is low.

To summarize, we have demonstrated binding and activation of Src family members to Tyr568 in Kit/SCFR and that this tyrosine phosphorylation site is necessary for phosphorylation of Shc and activation of the Ras/MAP kinase pathway, c-*fos* induction, while its role in SCF-induced mitogenicity is marginal. In contrast, simultaneous mutation of both Tyr568 and Tyr570 to phenylalanine residues leads to an almost complete loss of SCF-induced mitogenicity. This suggests that additional signal transduction molecules bind to Tyr568 and Tyr570, and that these are important for the mitogenic response to SCF. Future work will focus on the identification and cloning of additional signal transduction molecules binding to Tyr568 and Tyr570, that could explain the difference between Src activation and DNA synthesis in these mutant cell lines.

## Materials and methods

### *Antibodies, antisera, peptides and GST fusion proteins*

Recombinant human SCF was a kind gift of AMGEN. The rabbit antiserum Kit-C1, recognizing the C-terminal tail of the Kit/SCFR was purified as described by Blume-Jensen *et al*. (1993). The rabbit antiserum cst-1, recognizing c-Src, c-Yes and c-Fyn, was prepared as described by Kypta *et al*. (1990). A rabbit antiserum recognizing Erk-2, EET, was raised against the carboxyterminal sequence of Erk-2 (EETARFQPGYRS) (Leevers and Marshall, 1992). Affinity purified anti-Shc antibodies were purchased from Transduction Laboratories and the PY99 anti-phosphotyrosine antibody from Santa Cruz Biotechnology. A rabbit antiserum recognizing the juxtamembrane region of the Kit/SCFR (VVE) was raised against a synthetic peptide corresponding to amino acids 559–579 in the human Kit/SCFR (VVEINGNNYVYIDPTQLPYDH). The GST fusion protein containing the SH2 domain of c-Src was a kind gift from Dr Tony Pawson.

### *Site-directed mutagenesis and plasmid constructs*

Mutations were introduced using the Altered Sites™ *in vitro* mutagenesis system (Promega) according to the manufacturer's instructions, essentially as described (Blume-Jensen *et al*., 1995). Briefly, single-stranded DNA was produced from a fragment of the human c-*kit* cDNA cloned into the phagemid vector pALTER™-1. For mutagenesis, the following oligonucleotides were used: Tyr568 to Phe: GGA AAC AAT TTT GTT TAC ATA, Tyr570 to Phe: CAA TTA TGT TTT CAT AGA CCC A, and Tyr568 and Tyr570 to Phe: AAT GGA AAC AAT TTT GTT TTC ATA GAC CCA ACA. Colonies were screened for mutations by direct sequencing (Sequenase version 2.0 DNA dideoxynucleotide sequencing kit; US Biochemical Corporation). The mutated fragment was then subcloned into the full-length c-*kit* cDNA in the mammalian expression vector pSV7d (Truett *et al*., 1985). Finally, the mutated c-*kit* cDNA was cloned into pcDNA1/Neo, a cytomegalovirus-based expression vector.

### *Establishment of stable Kit/SCFR-expressing PAE cell lines*

Porcine aortic endothelial (PAE) cells (Miyazono *et al*., 1985) were transfected with the mutant constructs by electroporation and clonally expanded in the presence of geneticin, as described (Blume-Jensen *et al*., 1995).

### *Cell culture*

PAE cells expressing the Kit/SCFR were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg streptomycin and 2 mM L-glutamine.

### *Peptide synthesis and purification*

Peptide synthesis and purification were performed as described by Hansen *et al*. (1996). Peptides were stored under dry conditions at room temperature. Peptide stock

solutions were kept in 20 mM HEPES, pH 7.4 at −20°C. The following tyrosine phosphorylated peptides were synthesized: INGNNY(p)VYIDPTQLPYDHK (pY568 peptide), INGNNYVY(p)IDPTQLPYDHK (pY570 peptide) and INGNNY(p)VY(p)IDPTQLPYDHK (pY568/pY570 peptide). The corresponding non-phosphorylated peptides was also synthesized, INGNNYVYIDPTQLPDYHK (Ref peptide).

*Immunoprecipitation and Western blotting*

Immunoprecipitation and Western blotting was performed as described by Blume-Jensen *et al*. (1991).

*GST fusion protein binding assay*

PAE cells expressing the wild-type Kit/SCFR or the Y568F, the Y570F or the Y568F/Y570F mutants of the Kit/SCFR were stimulated or not with 100 ng/ml SCF for 5 min at 37°C, rinsed twice in ice-cold PBS, followed by lysis for 10 min on ice in lysis buffer (1% Triton X-100, 10% glycerol, 0.15 M NaCl, 20 mM HEPES, pH 7.4, 5 mM EDTA, 1% Trasylol, 400 $\mu$M $Na_3VO_4$ and 1 mM PMSF). The GST-Src SH2 domain fusion protein was pre-bound to glutathione-Sepharose beads (Pharmacia Biotech) for 30 min end-over-end at 4°C, and then washed three times in lysis buffer. Preassociated GST-Src SH2 fusion protein (2 $\mu$g) was added to each lysate in the presence or absence of 100 $\mu$M competitive peptide, and incubated end-over-end for 2 h at 4°C. The adsorbed material was washed three times in lysis buffer and once in 20 mM HEPES, pH 7.4. The samples were boiled for 5 min in reducing Laemmli buffer and proteins separated on a 7% SDS–PAGE gel. Separated proteins were transferred to an Immobilon P membrane (Millipore Corporation) and subjected to Western blotting using affinity purified KitC1 antibody, recognizing Kit/SCFR (Blume-Jensen *et al*., 1993).

*$^{32}$P-orthophosphate labeling of cell, trypsin digestion, immunoprecipitation of tryptic peptides, phosphoamino acid analysis and Edman degradation*

$^{32}$P-orthophosphate labeling of cells was performed as described by Blume-Jensen *et al*. (1993). After SDS–PAGE and electrotransfer to a nitrocellulose filter, proteins were digested with trypsin as described by Blume-Jensen *et al*. (1995). Immunoprecipitation of tryptic peptides was performed essentially according to Blume-Jensen *et al*. (1995), except that the antiserum VVE, against the juxtamembrane region of the Kit/SCFR was used. Phosphoamino acid analysis on immunoprecipitated material and Edman degradation was performed essentially as described by Blume-Jensen *et al*. (1995).

*[$^3$H]thymidine incorporation assay*

Assay for incorporation of [$^3$H]thymidine into TCA-precipitable material was performed as described by Blume-Jensen *et al*. (1991).

*Northern blotting*

RNA was isolated from PAE cells using the Promega SV Total RNA Isolation System according to the manufacturer's instructions. The RNA was separated on a 1% agarose gel. After transfer to Hybond N$^+$ (Amersham Life Science), RNA was UV-crosslinked to the membrane according to the manufacturer's instructions. The membrane was hybridized overnight at 65°C with $^{32}$P-labeled probes in 1.5 × SSPE, 10% PEG 6000, 7% SDS, 10 $\mu$g/ml of salmon sperm DNA. The filter was washed once in 1 × SSC and 0.1% SDS and then once in 0.5 × SSC and 0.1% SDS for 30 min at 65°C. The c-*fos* probe used was a *Hind*III-*Xba*I fragment (1.3 kb) and the $\beta$-actin probe (2.0 kb) was from Clontech. The probes were labeled using Rediprime random primer labeling kit (Amersham Life Science).

*Ras GTP-loading assay*

Subconfluent cell cultures were starved overnight in serum-free Ham's F-12. Measurement of Ras GTP-loading following SCF stimulation was done on $^{32}$P-orthophosphate labeled cells, essentially as described by Burgering *et al*. (1991). The amount of GTP and GDP that was bound to p21ras, was quantified using a Fuji BAS2000 Bioimager.

*MAP kinase assay*

Subconfluent cells were starved overnight in serum-free medium and stimulated or not with 100 ng/ml SCF at 37°C for the indicated time. Cells lysates were prepared, and immunoprecipitation performed using an antiserum against Erk2 (Leevers and Marshall, 1992). Erk2 kinase was assayed essentially according to Reuter *et al*. (1995), using myelin basic protein as a substrate. The amount of radioactivity incorporated into MBP was quantitated using a Fuji BAS2000 Bioimager.

*Src kinase assay*

Subconfluent cells were starved overnight in serum-free medium and stimulated with 100 ng/ml SCF at 37°C for 5 min, followed by lysis and immunoprecipitation with cst-1 antibodies and *in vitro* kinase assay using acid-denatured enolase as a substrate, as described by Kypta *et al*. (1990). Quantitation of radioactivity incorporated into enolase was done using a Fuji BAS2000 Bioimager.

In vitro *kinase assay using poly(Glu,Tyr) as an exogenous substrate*

Subconfluent cells were starved overnight in serum-free medium and stimulated with 100 ng/ml SCF at 37°C for 5 min, followed by lysis and immunoprecipitation with anti-Kit/SCFR antibodies. Immunoprecipitates were incubated with 50 $\mu$M $\gamma$[$^{32}$P]ATP and 20 $\mu$g poly(Glu,Tyr 4 : 1 (Sigma) in 40 $\mu$l of kinase buffer (10 mM $MnCl_2$, 20 mM HEPES, pH 7.4, 1 mM DTT) for 10 min at room temperature before separation by SDS-polyacrylamide electrophoresis and exposure to X-ray film.

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