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Src Family Kinases Are Involved in the Differential Signaling from Two Splice Forms of c-Kit*

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In both mice and humans alternate splicing results in isoforms of c-Kit characterized by the presence or the absence of a tetrapeptide sequence, GNNK, in the juxtamembrane region of the extracellular domain. Dramatic differences in the kinetics and magnitude of activation of the intrinsic tyrosine kinase activity of c-Kit between the GNNK− and GNNK+ isoforms has previously been shown. Here we report the analysis of downstream targets of receptor signaling, which revealed that the signaling was differentially regulated in the two splice forms. The kinetics of phosphorylation of Shc, previously demonstrated to be phosphorylated by Src downstream of c-Kit, was stronger and more rapid in the GNNK− form, whereas it showed slower kinetics in the GNNK+ form. Inhibition of Src family kinases with the specific Src family kinase inhibitor SU6656 altered the kinetics of activation of the GNNK− form of c-Kit so that it resembled that of the GNNK+ form. In cells expressing the GNNK− form, SCF was rapidly degraded, whereas in cells expressing the GNNK+ form only showed a very slow rate of degradation of SCF. In the GNNK+ form the Src inhibitor SU6656 only had a weak effect on degradation, whereas in the GNNK− form it dramatically inhibited degradation. In summary, the two splice forms, despite only a four-amino acid sequence difference, remarkable differences in their signaling capabilities.

The receptor for stem cell factor, c-Kit, is a type III receptor tyrosine kinase belonging to the same subfamily as the platelet-derived growth factor receptors, FLT3 (fms-like tyrosine kinase belonging to the same subfamily as the platelet-derived growth factor receptors), and the macrophage colony-stimulating factor receptor (1). The c-Kit gene is identical to the white spotting locus (W) in the mouse (2, 3). Partial or complete loss of function mutations in c-Kit result in macrocytic anemia, aberrations in pigmentmentation, decreased fertility, mast cell deficiency, reduction in gastrointestinal motility, and impairment in learning function in the hippocampus (reviewed in Refs. 4 and 5).

Ligand binding leads to activation of c-Kit, resulting in diverse cellular responses such as differentiation, proliferation, growth, survival, adhesion, and chemotaxis. These responses are the end result of the activation of multiple signal transduction pathways. Signal transduction molecules that bind to and become activated by c-Kit include Src family kinases (binding to Tyr668), Grb2 (binding to Tyr703 and Tyr936), SHP-2 (binding to Tyr568), SHP-1 (binding to Tyr577), and phosphoinositide 3-kinase (binding to Tyr722) (6–10).

Alternative mRNA splicing results in the production of two isoforms of c-Kit in the mouse and four in humans. In both mouse and human, alternate splicing results in isoforms characterized by the presence or absence of a four-amino acid sequence, GNNK, in the juxtamembrane region of the extracellular domain (Fig. 1) (11, 12). This has been shown to be due to the use of alternative 5′ splice donor sites (13, 14). The two splice forms, denoted GNNK− and GNNK+, respectively, are co-expressed in most tissues with the GNNK− form predominating (11, 12, 15). Caruana et al. (16) demonstrated that NIH3T3 cells expressing either isoform differed in their transforming activity. In the presence of SCF, the GNNK− form induced anchorage-independent growth, loss of contact inhibition, and tumorigenicity. No difference in ligand binding affinity was observed between the two isoforms. It was demonstrated that upon ligand stimulation, the GNNK− isoform was more highly tyrosine-phosphorylated, more rapidly internalized, and activated ERK more strongly than the GNNK+ isoform.

In this study, we have analyzed the molecular signaling mechanisms activated by the GNNK− and the GNNK+ isoforms. We have found that dramatic differences in activation of particular signal transduction pathways occur, whereas others remain identical between the two isoforms. The differences in signaling between the two isoforms were found to be to a large extent dependent on differential recruitment and activation of Src family kinases.

EXPERIMENTAL PROCEDURES

Antibodies, Antiserum, Peptides, and Glutathione S-Transferase Fusion Proteins—Recombinant human SCF was a kind gift of AMGEN, Inc. The Src inhibitor SU6656 was a kind gift of SUGEN, Inc. The rabbit antiserum Kit-C1, recognizing the C-terminal tail of c-Kit, was

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The abbreviations used are: SCF, stem cell factor; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline.
Splice Form-specific Signaling by c-Kit

RESULTS

Differences in Kinetics and Magnitude of Phosphorylation and the Rate of Degradation between the Two Splice Forms—NIH3T3 cells stably expressing either the GNNK+ form or the GNNK+ form of human c-Kit were stimulated with 100 ng/ml recombinant human SCF for the indicated times at 37 °C, lysed in Triton X-100 lysis buffer, and subjected to immunoprecipitation with c-Kit antibodies, followed by SDS gel electrophoresis and electrotransfer to an Immobilon-P membrane. All of the preparations of antibodies used in this paper gave strong signals in wild-type NIH3T3 and NIH3T3 cells as well as NIH3T3 cells transfected with c-Kit. There was no apparent receptor degradation (Fig. 2). In contrast, the GNNK+ form showed a slow kinetics of rather weak autophosphorylation that persisted over a long period of time with no apparent receptor degradation.

Shc Is Phosphorylated More Rapidly and with Higher Stoichiometry by the GNNK+ Form than by the GNNK+ Form—We have previously shown that Src is downstream of c-Kit, leading to phosphorylation of Shc, recruitment of Grb2-Sos, and activation of the Ras/mitogen-activated protein kinase pathway (8). Therefore, we wanted to investigate whether Shc was phosphorylated more rapidly and with higher stoichiometry by the GNNK+ form than by the GNNK+ form (Fig. 3A). Quantitation of data from CCD camera detection of chemiluminescence revealed an almost 3-fold higher magnitude of phosphorylation of Shc by the GNNK+ form compared with the GNNK+ form (Fig. 3B).
shown that phosphorylation of Shc by c-Kit is dependent on Src kinase activity (8), we wanted to investigate whether Src was activated differentially by the two c-Kit isoforms. NIH3T3 cells expressing either the GNNK- or the GNNK+ form of c-Kit were stimulated with SCF and lysed. The lysates were incubated with a glutathione S-transferase fusion protein of the SH2 domain of c-Src prebound to glutathione-Sepharose beads. The beads were washed extensively, and the bound material was separated on a SDS-PAGE, followed by electrotransfer to an Immobilon-P membrane. Probing of the membrane with Kit-C1 antibody revealed that the SCF-stimulated GNNK- isoform readily associated with the Src SH2 domain, whereas association of the GNNK+ form was very weak (Fig. 4A). To measure the kinase activity of Src, lysates from NIH3T3 cells expressing either the GNNK- or the GNNK+ form of c-Kit were subjected to immunoprecipitation with an antibody against c-Src and tested in an in vitro kinase assay using acid-denatured enolase as a substrate. As a control for specificity, the immunoprecipitates were incubated either with or without the Src kinase inhibitor SU6656. The GNNK- isoform could be demonstrated to induce a considerably stronger activation of c-Src than the GNNK+ isoform (Fig. 4B). To verify that the kinase activity of c-Kit was unaffected by SU6656, immunoprecipitates of c-Kit from SCF-stimulated cells were incubated with [γ-32P]ATP in the presence or absence of SU6656 (Fig. 4C). As expected, the kinase activity of c-Kit, as judged by autophosphorylation, was unaffected by SU6656. The lower activity seen in the GNNK- form can be explained by the rapid ligand-induced degradation of c-Kit, compared with the GNNK+ isoform. The lower band seen corresponds to the immature form of c-Kit.

**Phosphorylation of ERK Induced by the GNNK- Form of c-Kit Is Stronger than by the GNNK+ Form and Dependent on the Activity of Src Family**—When studying the signaling from the GNNK- and GNNK+ forms of c-Kit, Caruana et al. (16) demonstrated a dramatic difference in ERK activation between the splice forms. Whereas the GNNK- form was strongly and rapidly activated, the GNNK+ induced a weak activation of ERKs. One possible explanation for this difference in ERK activation could be that different degrees of phosphorylation of Shc by Src might lead to differences in ERK activation.

We showed that in NIH3T3 cells expressing the GNNK- form of c-Kit, the phosphorylation of ERK occurred much faster and reached a higher level than in cells expressing the GNNK+ form (Fig. 5). Furthermore, activation of ERKs could largely be inhibited by the Src inhibitor SU6656, suggesting a role of Src family kinases in the activation of ERKs by c-Kit in these cells.

*The Src Inhibitor SU6656 Causes a Decrease in Phosphorylation and Degradation of the GNNK- Form of c-Kit, as Compared with the GNNK+ Isoform*—The finding that c-Src associated more strongly with the GNNK- form than with the GNNK+ form, together with the finding that Shc, a substrate...
for c-Src, was phosphorylated to a higher degree by the GNNK form than the GNNK+ form, prompted us to investigate the role of c-Src in the differential signaling seen by the two isoforms of c-Kit.

Preincubation of NIH3T3 cells stably expressing the GNNK− form of c-Kit with the selective Src inhibitor SU6656 (22) caused slower kinetics of autophosphorylation of c-Kit, as well as a lower magnitude of autophosphorylation (Fig. 6). Furthermore, degradation of c-Kit in the presence of SU6656 was not as prominent as in the absence of inhibitor. When comparing the GNNK− form in the presence of Src inhibitor, the kinetics of phosphorylation and degradation resembled the pattern of the GNNK+ form, suggesting an involvement of differential activation of c-Src as a mechanism of splice-specific signaling.

**Kinetics of SCF-induced Internalization in the Two Splice Forms of c-Kit**—To assess whether the decrease in c-Kit expression following ligand stimulation of NIH3T3 cells expressing either the GNNK− form or the GNNK+ form was due to differences in internalization, the cells were incubated with 125I-SCF on ice for 1 h. The cells were washed extensively and transferred to 37 °C and incubated for the indicated period of time. The cell surface bound radioactivity was removed by washing with an acidic buffer. Ligand-induced internalization was only slightly faster in the GNNK− form compared with the GNNK+ form (data not shown), and by use of the SU6656 Src kinase inhibitor, it could be shown to be partially dependent on the activity of Src family kinases.

**Degradation of SCF Internalized through c-Kit Occurs Very Rapidly in Cells Expressing the GNNK− Form, whereas It Is Degraded Slowly in Cells Expressing the GNNK+ Form**—The cell lysates from the experiment described above were treated with 10% trichloroacetic acid to precipitate protein bound ra-
acid-soluble radioactivity was taken as a measure of degradation of
jected to precipitation with 10% trichloroacetic acid. Trichloroacetic
for the indicated periods of time. The medium was collected and sub-
ated with 125I-SCF for 60 min on ice, followed by incubation at 37

Given the dramatic differences in degradation of the GNNK
and positively regulate the ubiquitin ligase activity of Cbl (24).
ligase activity. Src has been demonstrated to phosphorylate
collaborators (23) demonstrated that Cbl possesses a ubiquitin E3
involved in regulation of polyubiquitination of proteins, which
has been demonstrated to tag proteins for degradation in either
the lysosomes or in the proteasome complex. Joazeiro and
colleagues (23) demonstrated that Cbl possesses a ubiquitin E3
ligase activity. Src has been demonstrated to phosphorylate and
positively regulate the ubiquitin ligase activity of Cbl (24). Given
the dramatic differences in degradation of the GNNK—
and GNNK+ isoform following SCF stimulation, we wanted to
test whether differences in Src-mediated phosphorylation of
Cbl could account for the differences in degradation. NIH3T3 cells
expressing either isoform were stimulated with SCF in the
presence or absence of SU6656, lysed, and subjected to immu-
noprecipitation with an antibody against Cbl. Immunoprecipi-
tated proteins were separated by SDS gel electrophoresis,
electrotransferred to an Immobilon-P filter, and probed with anti-
phosphotyrosine antibodies followed by reprobing with Cbl
antibodies. It could be shown that phosphorylation of Cbl was
considerably stronger in cells expressing the GNNK—
compared with cells expressing the GNNK+ isoform and that
the phosphorylation was inhibited by the Src inhibitor SU6656
(Fig. 8).

Mitogenic Response to SCF Is Stronger in NIH3T3 Cells
Expressing the GNNK— Form as Compared with the GNNK+ Form—It has previously been shown that the GNNK— form is better than the GNNK+ form in inducing anchorage-independent growth as well as focus formation of NIH3T3 cells (16). Furthermore, several of the above mentioned signal transduction pathways have been linked to transformation, such as the

Src family kinases and the Ras/ERK pathway. Therefore, we
compared the ability of the GNNK— and the GNNK+ forms to
mediate an SCF-dependent mitogenic response. NIH3T3 cells
expressing either isoform of c-Kit were starved for 24 h, fol-
lowed by stimulation with SCF for 48 h in the presence of
[3H]thymidine. Trichloroacetic acid-soluble radioactivity was
counted in a scintillation counter. A difference in mitogenic
response to SCF was observed, where the GNNK— form was
better than the GNNK+ form (Fig. 9). This is consistent with
the observations that the Src family kinases and the Ras/ERK
pathway are more efficiently activated by the GNNK— form.

Kinetics of Phosphorylation of Individual Tyrosine Residues
in c-Kit Follows Different Kinetics than the Overall Phospho-
ylation—To be able to study the phosphorylation of individual
residues in c-Kit, a panel of phosphospecific antibodies were
produced recognizing phosphorylated Tyr568 (c-Src associa-
tion site), Tyr721 (phosphoinositide 3-kinase association
site), Tyr921 (activation loop), and Tyr936 (association site for
Grb2 and Grb7). To verify the specificity of the individual
phosphospecific antibodies, COS7 cells were transfected with
either wild-type c-Kit (GNNK+) or the corresponding tyrosine-
to-phenylalanine mutation, stimulated with SCF for 10 min at
37 °C, lysed, and immunoprecipitated with a c-Kit antibody.
After electrophoresis and transfer to Immobilon P membranes,
the filters were probed with either phosphospecific antibody. In
all cases, wild-type c-Kit gave a strong signal, whereas the
corresponding tyrosine-to-phenylalanine mutant gave a weak
signal or no signal (Fig. 10A). To verify that the tyrosine kinase
activity of the mutant receptors was not impaired, the filters
were stripped and reprobed with anti-phosphotyrosine antibod-
ies (Fig. 10B).

NIH3T3 cells expressing either the GNNK— form or the
GNNK+ form were stimulated with 100 ng/ml SCF for the
indicated time periods, lysed, and subjected to immuno-
precipitation with the Kit-C1 antibody. Immunoprecipitated receptor
was separated by SDS-PAGE and electrotransferred to Immo-
bilon-P membrane. The filter was then probed with either
phosphotyrosine antibodies (PY99), TyrP568, TyrP721,
TyrP921, or TyrP936 antibodies, respectively (Fig. 11). As
previously demonstrated, ligand stimulation of the GNNK—
form led to rapid phosphorylation, ubiquitination, and degra-
dation of c-Kit, whereas the GNNK+ showed a slower kinetics,
weaker phosphorylation, but persistent signaling (Fig. 11).
When probing an identical filter with the TyrP568 antibody,
rapid and transient phosphorylation was seen in the GNNK—
at the 2-min time point but then rapidly declined. In contrast,
the GNNK\textsuperscript{+} form showed a constant low level of phosphorylation, even in the absence of SCF stimulation. The Tyr(P)\textsuperscript{823} antibody against the activation loop tyrosine thought to be of importance for activation of c-Kit kinase activity followed the same kinetics as the overall tyrosine phosphorylation of c-Kit; in the GNNK\textsuperscript{+} form there was a rapid and strong increase in phosphorylation, whereas in the GNNK\textsuperscript{−} form the phosphorylation was slow, but persisted for a longer time, and was of the same magnitude as for the GNNK\textsuperscript{−} form. This is in agreement with previous observations by Caruana \textit{et al.} (16), demonstrating a similar magnitude in association of phosphoinositide 3-kinase to c-Kit as well as activation of Akt by the two splice forms. Phosphorylation of Tyr\textsuperscript{936}, constituting a docking site for both Grb2 and Grb7, showed strong and rapid kinetics in the GNNK\textsuperscript{+} form, whereas it was even stronger and more persistent in the GNNK\textsuperscript{−} form (Fig. 11). In summary, phosphorylation of individual sites did not follow the same kinetics as the overall phosphorylation, which could explain why different signal transduction pathways are affected to different extent by the two splice forms.

**DISCUSSION**

Several splice forms of human c-Kit have been described to date (11, 12), but the physiological significance of these different forms is unknown. Two of the existing splice forms differ in the presence or absence of a four-amino acid stretch, GNNK. To investigate the function of these isoforms, we have used NIH3T3 fibroblasts, which lack endogenous c-Kit, transfected with either splice form of c-Kit (16). It was previously shown that NIH3T3 cells expressing the GNNK\textsuperscript{+} form exhibited SCF-dependent anchorage independent growth and loss of contact inhibition and were tumorigenic in nude mice (16). In contrast, NIH3T3 cells expressing the GNNK\textsuperscript{−} form only a very weak signal was detected. Probing with the Tyr(P)\textsuperscript{721} antibody, which detects phosphorylation of the phosphoinositide 3-kinase association site in c-Kit, revealed a rapid and strong phosphorylation in the GNNK\textsuperscript{−} form, whereas in the GNNK\textsuperscript{+} the phosphorylation was slow, but persisted for a longer time, and was of the same magnitude as for the GNNK\textsuperscript{−} form. This is in agreement with previous observations by Caruana \textit{et al.} (16), demonstrating a similar magnitude in association of phosphoinositide 3-kinase to c-Kit as well as activation of Akt by the two splice forms. Phosphorylation of Tyr\textsuperscript{936}, constituting a docking site for both Grb2 and Grb7, showed strong and rapid kinetics in the GNNK\textsuperscript{−} form, whereas it was even stronger and more persistent in the GNNK\textsuperscript{+} form (Fig. 11). In summary, phosphorylation of individual sites did not follow the same kinetics as the overall phosphorylation, which could explain why different signal transduction pathways are affected to different extent by the two splice forms.
splice forms. We could show that the kinetics of phosphorylation of c-Kit was much faster and stronger in the GNNK – form, compared with the GNNK+ form (Fig. 2). This was followed by a rapid degradation of the GNNK – form, whereas expression of the GNNK+ form remained stable. Yee et al. (25) showed that in murine mast cells, ligand binding to c-Kit leads to rapid internalization and ubiquitin-mediated degradation. Inactivation of the receptor kinase resulted in reduced rate of internalization of ligand-receptor complexes, and no ubiquitination took place. However, Gommerman et al. (26) found, when studying the murine lymphoma cell line DA-1 transfected with c-Kit, a dependence of an intact Tyr724 site for internalization of ligand bound c-Kit. A partial role for Src family kinases in ligand-driven internalization of c-Kit was demonstrated by Broudy et al. (27) using the inhibitor PP1. In our hands, inhibition of Src family kinases caused a partial inhibition of c-Kit internalization, but a dramatic effect was seen on ligand degradation (Fig. 8). A role for c-Src in receptor degradation has been seen for platelet-derived growth factor α-receptor (28). It was shown that receptor-mediated phosphorylation of c-Cbl and polyubiquitination of the receptor required the association site for Src family kinases on the receptor. In other words, ligand-induced activation of Src family kinase activity was a prerequisite for c-Cbl phosphorylation and ubiquitination. It has recently been demonstrated that c-Cbl is a ubiquitin E3 ligase (23).

We and others have previously shown (8, 29) that the main pathway of c-Kit activation of the Ras/ERK pathway goes through Src-dependent phosphorylation of Shc. The Y568F/ Y570F mutant of c-Kit, which fails to activate Src family kinases, mediates only a very weak activation of ERK. The two autophosphorylation sites, Tyr703 and Tyr936, have been shown to bind to Grb2 in vitro (10) seem to be of minor importance in the in vivo situation. The stoichiometry of Tyr703 phosphorylation has been shown to be quite low.2 Furthermore, the low degree of direct association of Grb2 to c-Kit is lost if Tyr703 is mutated to phenylalanine,2 suggesting that Tyr703 plays a minor role in binding of Grb2 in the in vivo situation. In this paper, we show that c-Kit-mediated activation of ERK is stronger in the GNNK – form compared with the GNNK+ form, correlating with an increased degree of activation of Src family kinases by the GNNK – form compared with the GNNK+ form. Furthermore, activation of ERK by the two isoforms of c-Kit is inhibited by the Src inhibitor SU6656 (Fig. 5). Thus, it is likely that the differences in activation of the Ras/ERK pathway can be accounted for by the differential regulation of Src family kinases by the two splice forms. The role of ERK activation in the mitogenic response to c-Kit stimulation is unclear. We have previously shown (8) that when the two tyrosine residues responsible for activation of Src family kinases, Tyr68 and Tyr770 in c-Kit, are mutated and the level of ERK activation is very low, we still get a reasonable mitogenic response. Other studies have also shown that, under certain circumstances, c-Kit-mediated mitogenesis can be independent of ERK activation (29).

The rapid and strong autophosphorylation of c-Kit seen in the GNNK – form could be due to c-Src-mediated phosphorylation of c-Kit at Tyr724 in the activation loop of the tyrosine kinase domain. Our data show that phosphorylation of Tyr724 parallels that of total receptor autophosphorylation. Tyr724 in the activation loop is conserved in most tyrosine kinases and in many cases found to be of importance for regulation of its activity in, for example, the insulin receptor and the fibroblast growth factor receptor (30, 31). However, cells expressing the Y823F mutant of c-Kit still show a level of kinase activity similar to wild-type receptors.2 Using cells overexpressing either kinase active or inactive c-Src, Biscardi et al. (32) showed that c-Src mediates phosphorylation of the epidermal growth factor receptor at Tyr845 and Tyr1101. Tyr845 is located in a position analogous to Tyr724 in c-Kit, and phosphorylation of this site by c-Src was found to contribute positively to epidermal growth factor-mediated mitogenesis.

It is striking that a stretch of only four amino acids in the juxtamembrane region of the extracellular domain could make such a dramatic difference in the signaling characteristics of a receptor. However, recent data suggest that not only ligand-induced dimerization is required for full activation of receptor tyrosine kinases but also the steric orientation of the two receptor subunits (for review, see Ref. 33). It has been shown that full activation of the erythropoietin receptor requires not only dimerization of receptors but also the correct orientation of the receptor subunit (34). Recently, Leibiger et al. (35) described differences in the signal transduction pathways activated and the repertoire of gene expression induced upon stimulation of the two splice forms of the insulin receptor, INSR-A and INSR-B. The INSR-A isoform was shown to induce expression of the insulin gene, whereas the INSR-B isoform mediated induction of glucokinase. Through differential use of exon11, the two splice forms of the insulin receptor differ in only 12 twelve amino acids in the C terminus of the α-subunit. Furthermore, Bell et al. (36) made a series of platelet-derived growth factor β-receptor mutants in which they put a dimerization motif derived from the sequence of oncogenic Neu at different positions in the transmembrane region. This led to the creation of a series of constitutive receptor dimers in which the intracellular parts were gradually rotated 103° for each mutant. Interestingly, rotational linkage of the transmembrane domain with the kinase domain was evidenced by a periodic activation of the receptor because the dimerization motif was shifted across the transmembrane domain. It might very well be that the four-amino acid insert, which is located in a predicted α-helical region, leads to a less favorable positioning of the intracellular parts of the receptor in a dimer than if those amino acids are absent. Interestingly, a similar pair of splice variants affecting the juxtamembrane region of the extracellular domain have been described for the ErbB2 receptor (37). A novel transcript of ErbB2 was found in human carcinomas, involving a deletion of 16 amino acids in the juxtamembrane region of the extracellular domain. It was shown that this splice form showed much stronger tyrosine kinase activity than the normal splice form, and it was also much stronger in its transforming ability, as judged by focal formation assay. Finally, Moriki et al. (38) demonstrated the importance of correct orientation of the epidermal growth factor receptors in a dimer for full kinase activity.

Future studies are aimed at understanding the precise mechanism by which these two splice forms can activate Src family kinases and other signal transduction pathways differently. By using cDNA microarray technology, we will be able to dissect in greater detail the differences and similarities in gene induction/repression by the different splice forms of c-Kit and attempt to link these observations to differences in biological responses induced by the two receptor forms.

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