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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 show, 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 3), 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 pigmentation, 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 Tyr^{568}), Grb2 (binding to Tyr^{703} and Tyr^{936}), SHP-2 (binding to Tyr^{568}), SHP-1 (binding to Tyr^{570}), and phosphoinositide 3-kinase (binding to Tyr^{721}) (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,[1] 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, Antisera, 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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[1] The abbreviations used are: SCF, stem cell factor; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline.

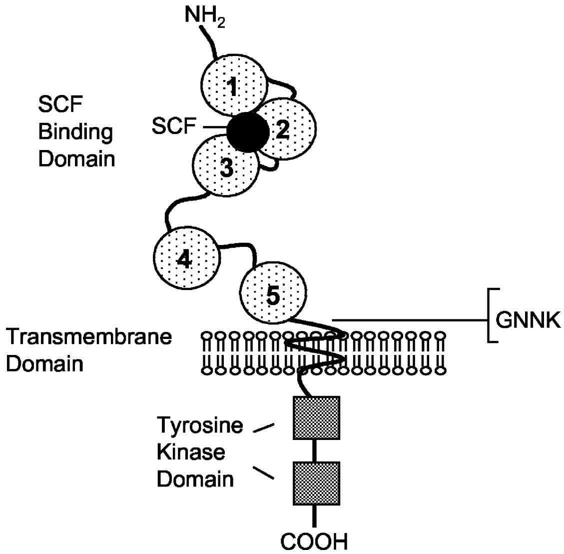

FIG. 1. **Schematic outline of the architecture of c-Kit.** The extracellular part of c-Kit consists of five immunoglobulin-like domains. Close to the plasma membrane, in the extracellular part of c-Kit, resides the sequence GNNK, which is either present or absent in the GNNK+ and GNNK− splice forms of c-Kit, respectively. The intracellular part of c-Kit contains the tyrosine kinase domain, which is split into two parts by the kinase insert sequence.

purified as described (17). A rabbit antiserum recognizing ERK2, EET, was raised against the C-terminal sequence of ERK2 (EETARFQPGYRS) (18). The Src antibody Ab-1 was from Oncogene Sciences. The anti-phosphotyrosine antibody PY99 and the Cbl antibody were from Santa Cruz Biotechnologies (Santa Cruz, CA), and affinity-purified anti-Shc antibodies were purchased from BD Transduction Laboratories; phospho-ERK (Thr^{202}/Tyr^{204}) was from Cell Signaling Technology. The glutathione *S*-transferase fusion protein of the c-Src SH2 domain was a kind gift from Dr. Tony Pawson.

Phosphospecific Antibodies—Phosphospecific antibodies against individual tyrosine phosphorylation sites in c-Kit were raised by immunizing rabbits with the following synthetic peptides conjugated to keyhole limpet hemocyanin by use of m-maleimidobenzoyl-*N*-hydroxysuccinimide ester: CEEINGNNpYVYIDPTQ ($Tyr(P)^{568}$), CSDSTNEpYMDMKPGV ($Tyr(P)^{721}$), CKNDSNpYVVKGA ($Tyr(P)^{823}$), and STNHIpYSNLANCS ($Tyr(P)^{936}$). The antibodies were extensively purified by chromatography over immobilized nonphosphorylated peptide, over phosphotyrosine-Sepharose, and finally over immobilized phosphopeptide. Purified antibodies were stored in 0.15 M NaCl, 20 M HEPES, pH 7.4, 50% glycerol at −70 °C. The specificity of each antibody was tested by Western blotting. COS7 cells were transiently transfected using LipofectAMINE (Roche Molecular Biochemicals) with either wild-type c-Kit or the corresponding tyrosine-to-phenylalanine mutant of c-Kit in pcDNA3 (Invitrogen). The cells were stimulated with 100 ng/ml of recombinant human SCF for 10 min, lysed, 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 receptors, whereas it gave no signal or a very weak signal in the corresponding tyrosine-to-phenylalanine mutant.

Cell Culture—NIH3T3 cells stably expressing c-Kit have previously been described (16). Pools of cells expressing equal levels of c-Kit were used throughout the study. NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Immunoprecipitation and Western Blotting—Immunoprecipitations and Western blotting were performed according to Ref. 17. Briefly, after stimulation of cells with 100 ng/ml SCF for the indicated period of time, the cells were washed once with ice-cold PBS, and lysed in a lysis buffer containing 1% Triton X-100, 25 mM Tris, pH 7.5, 1 mM Na_3VO_4, 1% Trasylol, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA. The lysates were centrifuged at 14 000 × *g* for 10 min at 4 °C, precleared for 30 min with 50 μl of a 1:1 slurry of protein A-Sepharose, and then incubated with the indicated antibody for 2 h at 4 °C. The immunoprecipitates were collected on protein A-Sepharose beads, washed three times in lysis buffer supplemented with 500 mM NaCl, and finally washed once with water. The samples were boiled for 3 min in reducing SDS sample buffer and separated by SDS-PAGE in 7% continuous gels, after which proteins were quantitatively electrotransferred to Immobilon P (Millipore) membranes. The membranes were blocked overnight in the cold with 0.2% Tween 20 in PBS, or in the case of phosphospecific antibodies, in 1% dry milk powder, 0.2% Tween 20 in PBS. The filters were incubated with primary antibody for 2 h at room temperature, followed by extensive washing with 0.5% Tween 20 in PBS. Incubation with secondary horseradish peroxidase-conjugated antibody was done for 1 h at room temperature, followed by extensive washing with 0.5% Tween 20 in PBS. The immunodetection was performed by enhanced chemiluminescence using the Super Signal Dura reagent from Pierce.

In Vitro Kinase Activity of c-Src and c-Kit—*In vitro* kinase assays of c-Src and c-Kit were performed as described (8). In brief, 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 Kit-C1 antibody or Src antibody Ab-1 (Oncogene Research), as outlined above. The immunoprecipitates were incubated with 50 μM [γ-^{32}P]ATP in 40 μl of kinase buffer (10 mM $MnCl_2$, 20 mM HEPES, pH 7.4, 1 mM dithiothreitol) for 10 min at room temperature before separation by SDS gel electrophoresis and exposure to x-ray film. Acid denatured enolase was used as an exogenous substrate of Src (19).

Internalization Experiments—Recombinant human SCF was labeled with ^{125}I as described (17). Internalization experiments were performed essentially as described (20). In brief, confluent cells in 12-well plates were incubated with 50,000 cpm of ^{125}I-SCF/well for 60 min on ice. After washing twice with binding medium (Dulbecco's modified Eagle's medium containing 1 mg/ml bovine serum albumin), the cells were incubated at 37 °C in binding medium for different time periods. The binding medium was removed and precipitated with an equal volume of 10% trichloroacetic acid. The amount of non-trichloroacetic acid-precipitable radioactivity was taken as an estimate of ligand degradation. After removal of the medium, the cells were washed in binding medium and incubated for 5 min on ice with phosphate-buffered saline containing 1 mg/ml bovine serum albumin adjusted to pH 3.7 with acetic acid. This acid wash procedure releases more than 90% of cell surface-bound ligand into the buffer. After treatment with acidic buffer, the cells were lysed in 1% Triton X-100. Radioactivity was determined using a Packard γ-counter.

[^{3}H]Thymidine Incorporation Assay—The assay for incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable material was performed as described (21).

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 using the Kit-C1 antibody. The immunoprecipitated proteins were separated by 7% SDS-PAGE, followed by electrotransfer to Immobilon-P. The filter was first probed with phosphotyrosine antibodies (PY-99), followed by stripping and reprobing with Kit-C1 antibody. It could be seen that the GNNK− form was strongly phosphorylated within minutes, followed by rapid 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 activated 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 we could detect differences in Shc phosphorylation by the two splice forms. Surprisingly, Shc was phosphorylated more rapidly and with higher stoichiometry by the GNNK− form than by the GNNK+ form (Fig. 3*A*). 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. 3*B*).

Stimulation of the GNNK− Form of c-Kit Leads to Association with and Activation of Src—Because we have previously

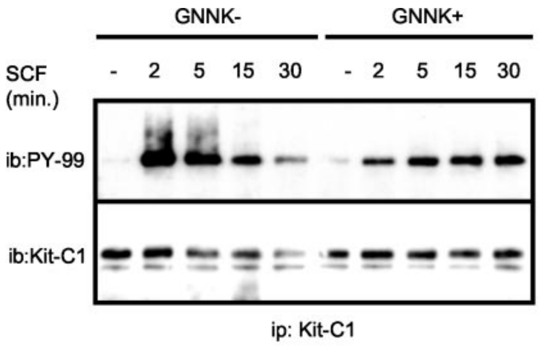

FIG. 2. **Kinetics of SCF-stimulated autophosphorylation in the GNNK− and the GNNK+ isoform, respectively.** NIH3T3 cells stably expressing either isoform of c-Kit were stimulated with 100 ng/ml of recombinant human SCF for the indicated periods of time. The cells were lysed and subjected to immunoprecipitation with the Kit-C1 antibody, followed by SDS gel electrophoresis and electrotransfer of proteins to an Immobilon-P filter. After blocking, the filter was probed with either phosphotyrosine antibodies (*PY-99*) and after stripping reprobed with Kit-C1 antibodies (*Kit-C1*). *ib*, immunoblot.

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. 4*A*). 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. 4*B*). 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-^{32}P]ATP in the presence or absence of SU6656 (Fig. 4*C*). 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

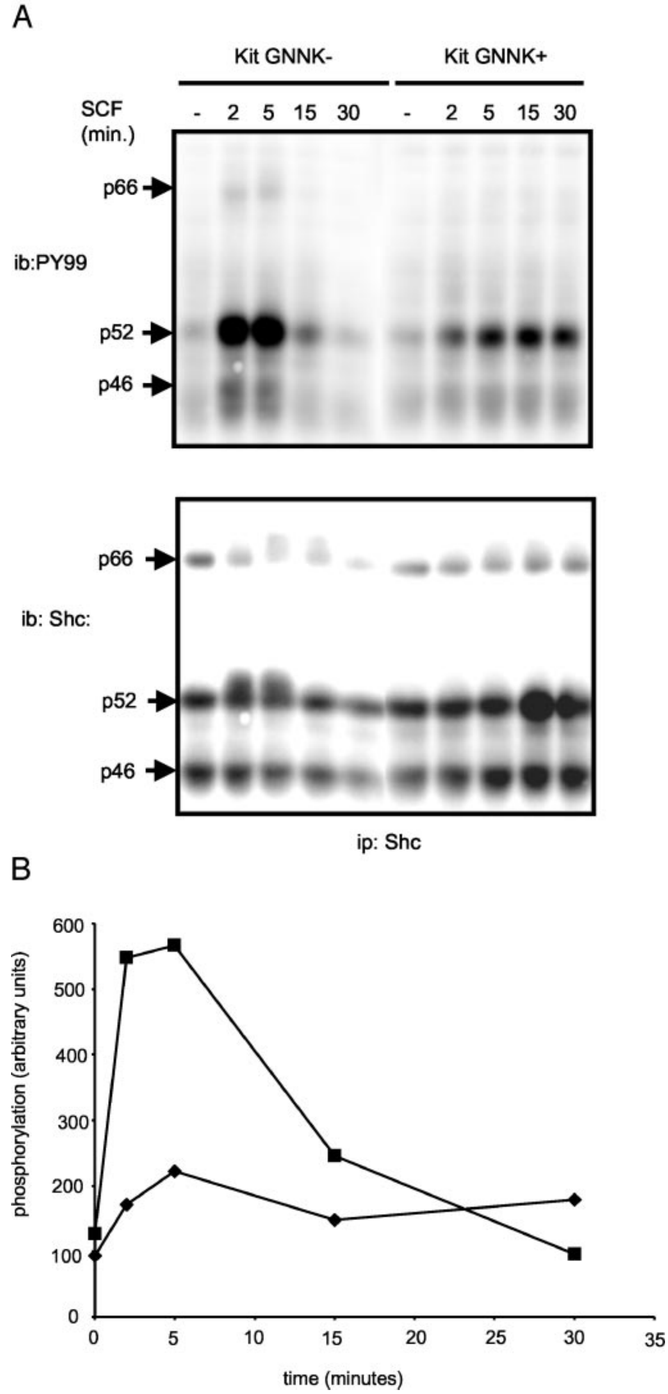

FIG. 3. **Kinetics of phosphorylation of Shc.** *A*, NIH3T3 cells stably expressing either isoform of c-Kit were stimulated with 100 ng/ml of recombinant human SCF for the indicated periods of time. The cells were lysed and subjected to immunoprecipitation with Shc antibodies, followed by SDS gel electrophoresis and electrotransfer of proteins to Immobilon-P filters. After blocking, the filter was probed with anti-phosphotyrosine antibodies (*PY99*), followed by stripping and reprobing with Shc antibodies. *B*, quantitation of data captured by Fuji LAS 2000 CCD camera. The intensity of the phosphotyrosine signal has for each *lane* been normalized with the signal of Shc protein. The degree of phosphorylation is given in arbitrary units. ■, GNNK− form of c-Kit; ♦, GNNK+ form of c-Kit. *ib*, immunoblot.

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

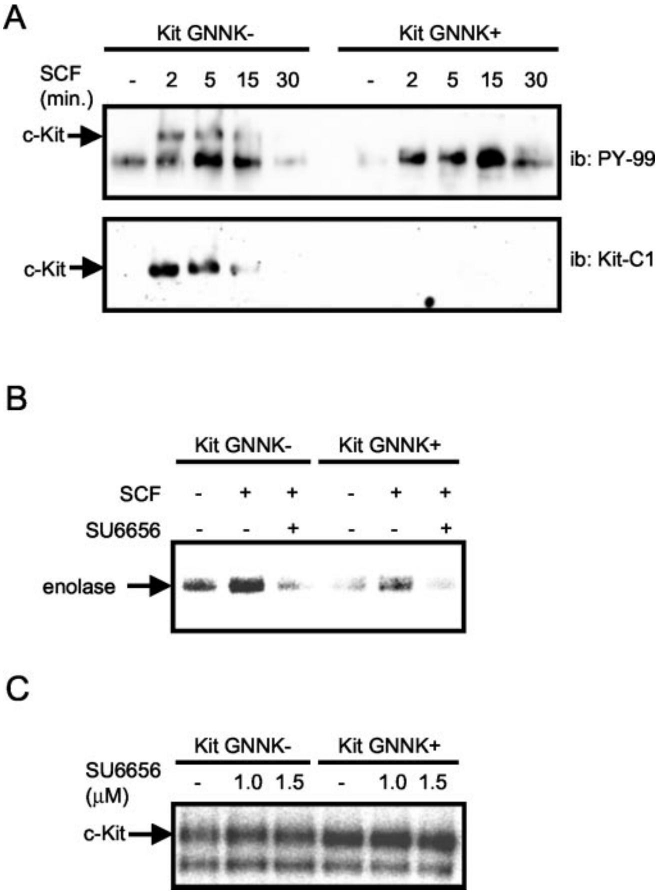

FIG. 4. **Differential association and activation of Src by the two c-Kit splice forms.** *A*, NIH3T3 cells expressing either isoform of c-Kit were stimulated with 100 ng/ml recombinant human SCF for the indicated periods of time. The cell lysates were prepared and incubated with 2 μg of glutathione *S*-transferase Src SH2 domain fusion protein preadsorbed to glutathione-Sepharose beads for 1 h end-over-end. After extensive washing, the bound proteins were resolved on SDS gel electrophoresis, followed by electrotransfer to Immobilon-P and probing with either anti-phosphotyrosine antibodies or with the Kit-C1 antibody. *B*, NIH3T3 cells expressing either isoform of c-Kit were preincubated with or without 2 μM SU6656 for 30 min, followed by stimulation with 100 ng/ml recombinant human SCF for 10 min. The cell lysates were prepared and subjected to immunoprecipitation with an antibody against c-Src, followed by incubation with [γ-^{32}P]ATP and acid-denatured enolase. Radiolabeled proteins were separated by SDS gel electrophoresis, electrotransferred to Immobilon P, followed by treatment with 1 M KOH for 1 h at 55 °C. The filter was exposed on a Fuji Imaging Plate and scanned in a Fuji BAS2000 Image Analyser. *C*, NIH3T3 cells expressing either isoform of c-Kit were stimulated with 100 ng/ml recombinant human SCF for 10 min. The cell lysates were prepared and subjected to immunoprecipitation with an antibody against c-Kit, followed by incubation with [γ-^{32}P]ATP and in the presence or the absence of the indicated concentration of the Src kinase inhibitor SU6656. Radiolabeled proteins were separated by SDS gel electrophoresis and electrotransferred to Immobilon P, followed by treatment with 1 M KOH for 1 h at 55 °C. The filter was exposed on a Fuji Imaging Plate and scanned in a Fuji BAS 2000 Image Analyser. *ib*, immunoblot.

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.

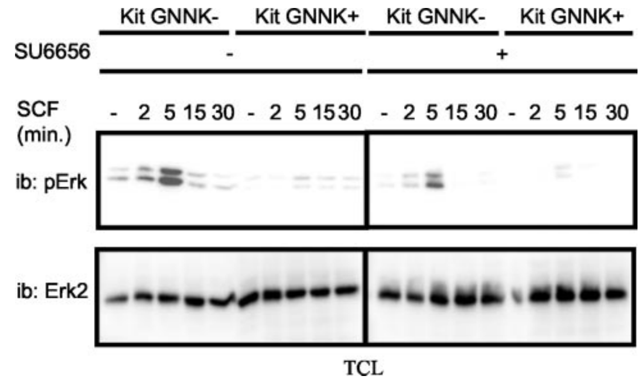

FIG. 5. **Kinetics and dependence of Src activity for activation of ERK1/2.** NIH3T3 cells stably expressing either isoform of c-Kit were stimulated with 100 ng/ml of recombinant human SCF for the indicated periods of time in the presence or absence of 2 μM SU6656. The cell lysates were prepared and separated by SDS gel electrophoresis, followed by electrotransfer to Immobilon-P filters. After blocking, the filter was probed with phospho-ERK antibodies (*pERK*), followed by stripping and reprobing with an antibody against ERK2. TCL, total cell lysate; *ib*, immunoblot.

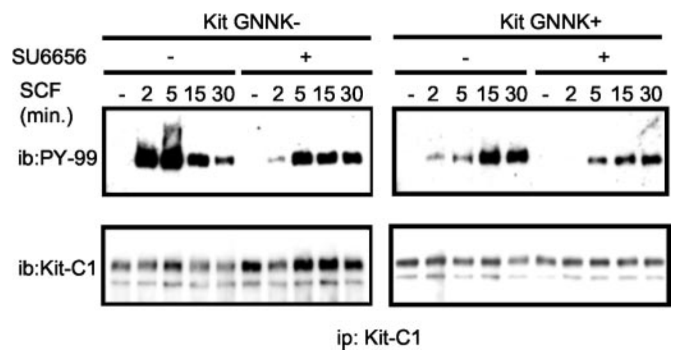

FIG. 6. **Effect of the Src-specific inhibitor SU6656 on the kinetics and magnitude of phosphorylation of c-Kit following ligand stimulation.** NIH3T3 cells expressing either isoform of c-Kit were preincubated or not with 2 μM SU6656 for 30 min at 37 °C. The cells were stimulated with 100 ng/ml recombinant human SCF for the indicated periods of time, lysed, and subjected to immunoprecipitation with the Kit-C1 antibody. Immunoprecipitated protein was separated by SDS gel electrophoresis, followed by electrotransfer to an Immobilon-P filter and probing of the filter with phosphotyrosine antibodies (*PY-99*), followed by stripping and reprobing with Kit-C1 antibodies (*Kit-C1*). *ib*, immunoblot; *ip*, immunoprecipitation.

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 ^{125}I-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 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-

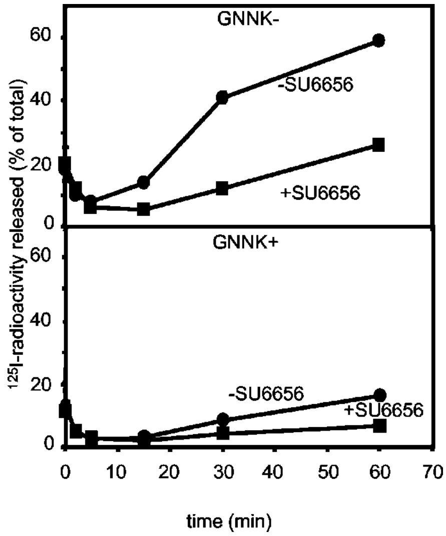

FIG. 7. **Kinetics of degradation of internalized SCF; comparison of the two isoforms of c-Kit and dependence of Src kinase activity.** NIH3T3 cells expressing either isoform of c-Kit were incubated with ^{125}I-SCF for 60 min on ice, followed by incubation at 37 °C for the indicated periods of time. The medium was collected and subjected to precipitation with 10% trichloroacetic acid. Trichloroacetic acid-soluble radioactivity was taken as a measure of degradation of ^{125}I-SCF. The experiments were carried out in the presence (■) or absence (●) of 2 μM SU6656.

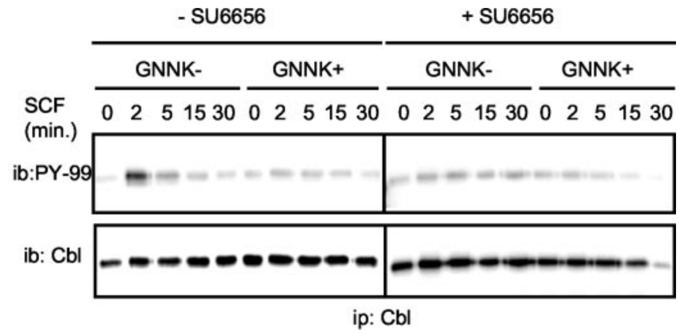

FIG. 8. **The GNNK− form of c-Kit mediates a stronger phosphorylation of Cbl than the GNNK+ form.** NIH3T3 cells expressing either isoform of c-Kit were preincubated with or without 2 μM SU6656 followed by stimulation with SCF. The lysates were prepared and subjected to immunoprecipitation using an antibody against Cbl. Immunoprecipitated proteins were separated by SDS gel electrophoresis and electrotransferred to Immobilon P. After blocking, the filter was probed with phosphotyrosine antibodies (*PY-99*), followed by reprobing with a Cbl antibody (*Cbl*). *ib*, immunoblot; *ip*, immunoprecipitation.

dioactivity. After centrifugation, the supernatant was counted in a γ-counter, as a measure of degradation. It could clearly be seen that degradation of ^{125}I-SCF bound to the GNNK+ form (Fig. 7) occurred very slowly and was slightly dependent on the activity of Src family kinases. In contrast, degradation of ^{125}I-SCF bound to the GNNK− form was very rapid, and degradation was inhibited to about 50% by the Src inhibitor SU6656.

Cbl Is Phosphorylated following SCF Stimulation of NIH3T3 Cells Expressing the GNNK− Form of c-Kit, whereas the GNNK+ Form Mediates Only a Weak Phosphorylation of Cbl—The multifunctional adapter protein Cbl has been shown to be 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 immunoprecipitation with an antibody against Cbl. Immunoprecipitated 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, followed by stimulation with SCF for 48 h in the presence of [^{3}H]thymidine. Trichloroacetic acid-precipitable 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 Phosphorylation—To be able to study the phosphorylation of individual tyrosine residues in c-Kit, a panel of phosphospecific antibodies were produced recognizing phosphorylated Tyr^{568} (c-Src association site), Tyr^{721} (phosphoinositide 3-kinase association site), Tyr^{823} (activation loop), and Tyr^{936} (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. 10*A*). To verify that the tyrosine kinase activity of the mutant receptors was not impaired, the filters were stripped and reprobed with anti-phosphotyrosine antibodies (Fig. 10*B*).

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 immunoprecipitation with the Kit-C1 antibody. Immunoprecipitated receptor was separated by SDS-PAGE and electrotransferred to Immobilon-P membrane. The filter was then probed with either phosphotyrosine antibodies (PY99), Tyr(P)^{568}, Tyr(P)^{721}, Tyr(P)^{823}, or Tyr(P)^{936} antibodies, respectively (Fig. 11). As previously demonstrated, ligand stimulation of the GNNK− form led to rapid phosphorylation, ubiquitination, and degradation of c-Kit, whereas the GNNK+ showed a slower kinetics, weaker phosphorylation, but persistent signaling (Fig. 11). When probing an identical filter with the Tyr(P)^{568} antibody, rapid and transient phosphorylation was seen in the GNNK− at the 2-min time point but then rapidly declined. In contrast,

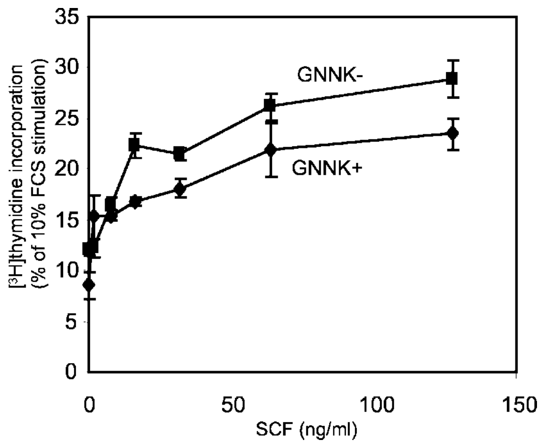

FIG. 9. **The GNNK− form of c-Kit mediates a stronger mitogenic response than the GNNK+ form.** NIH3T3 cells expressing either the GNNK− form (■) or the GNNK+ form (♦) of c-Kit were starved for 24 h in 1% bovine serum albumin. The cells were then stimulated with the indicated concentration of SCF for 48 h in the presence of [^{3}H]thymidine. The amount of [^{3}H]thymidine incorporated into DNA was determined in a scintillation counter. *FCS*, fetal calf serum.

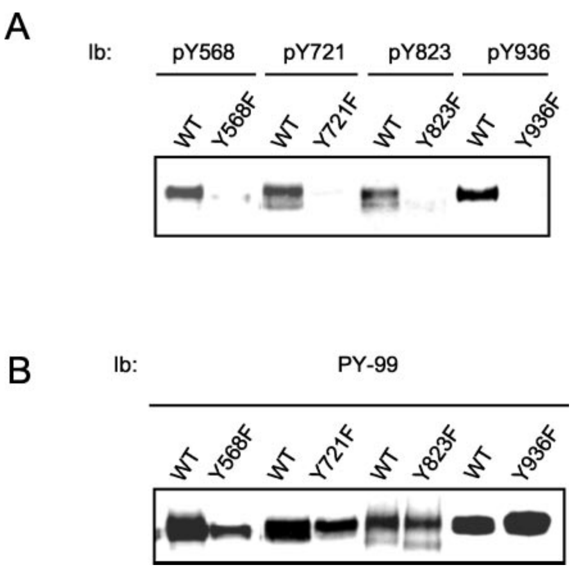

FIG. 10. **Phosphospecific antibodies directed against various phosphorylated tyrosine residues in c-Kit.** COS7 cells were transiently transfected with either wild-type c-Kit (GNNK+) or Y568F, Y721F, Y823F, or Y936F mutant form of c-Kit. The cells were stimulated with 100 ng/ml SCF for 10 min, followed by lysis and immunoprecipitation of c-Kit with Kit-C1 antibody. After SDS gel electrophoresis and subsequent electrotransfer to Immobilon P, the filters were blocked overnight and probed with the corresponding phosphospecific antibody (*A*) and stripped and reprobed with anti-phosphotyrosine antibodies (*B*). The blots were developed using Super Signal Dura reagent (Pierce). *Ib*, immunoblot; *WT*, wild type.

the GNNK+ form showed a constant low level of phosphorylation, even in the absence of SCF stimulation. The Tyr(P)^{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− form there was a rapid and strong increase in phosphorylation, whereas in the GNNK+ form only a very weak signal was detected. Probing with the Tyr(P)^{721} antibody, which detects phosphorylation of the phosphoinositide 3-kinase association site in c-Kit, revealed a rapid and strong phosphorylation in the GNNK− form, whereas in the GNNK+ the phosphorylation was slow, but persisted for a longer time, and was of the same magnitude as for the GNNK− form. This is in

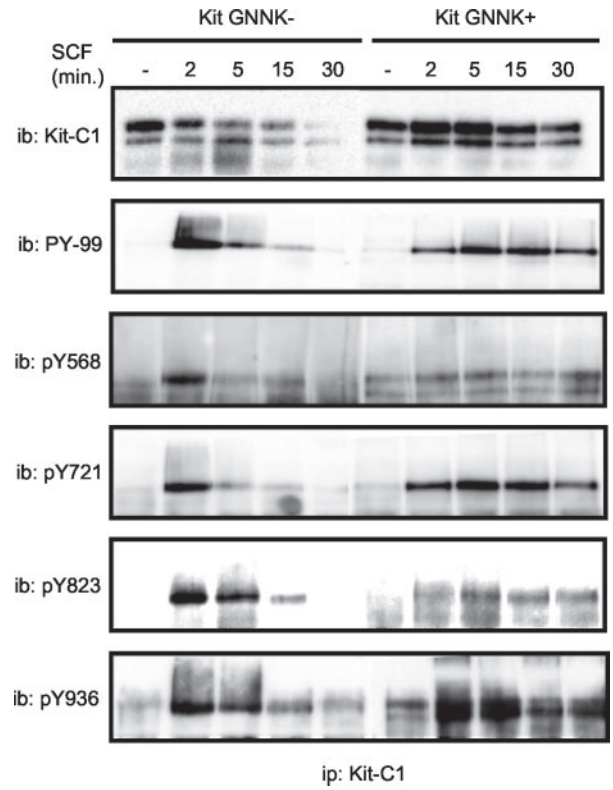

FIG. 11. **Kinetics and magnitude of phosphorylation of individual tyrosine residues in c-Kit.** NIH3T3 cells stably expressing either isoform of c-Kit were stimulated with 100 ng/ml of recombinant human SCF for the indicated periods of time. The cells were lysed and subjected to immunoprecipitation with Kit-C1 antibodies, followed by SDS gel electrophoresis and electrotransfer of proteins to Immobilon-P filter. After blocking, the filter was probed either with anti-phosphotyrosine antibodies (*PY-99*), Tyr(P)^{568} antibody, Tyr(P)^{721} antibody, Tyr(P)^{823} antibody, or Tyr(P)^{936} antibody. *ib*, immunoblot; *ip*, immunoprecipitation.

agreement with previous observations by Caruana *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^{936}, constituting a docking site for both Grb2 and Grb7, showed strong and rapid kinetics in the GNNK− form, whereas it was even stronger and more persistent in the GNNK+ 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 fibroblasts expressing the GNNK− 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+ exhibited anchorage-independent growth but relatively poor focus formation and did not form tumors in nude mice. Despite similar affinity for SCF, the GNNK− form displayed more rapid and extensive tyrosine autophosphorylation and faster internalization.

In this paper, we have studied the molecular mechanisms behind the differences in signal transduction from the two 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 Tyr^{721} 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, Tyr^{703} and Tyr^{936}, that have been shown to bind to Grb2 *in vitro* (10) seem to be of minor importance in the *in vivo* situation. The stoichiometry of Tyr^{703} phosphorylation has been shown to be quite low.[2] Furthermore, the low degree of direct association of Grb2 to c-Kit is lost if Tyr^{703} is mutated to phenylalanine,[2] suggesting that Tyr^{936} 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, Tyr^{568} and Tyr^{570} 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 Tyr^{823} in the activation loop of the tyrosine kinase domain. Our data show that phosphorylation of Tyr^{823} parallels that of total receptor autophosphorylation. Tyr^{823} 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 Tyr^{845} and Tyr^{1101}. Tyr^{845} is located in a position analogous to Tyr^{823} in c-Kit, and phosphorylation of this site by c-Src was found to contribute positively to epidermal growth factor-mediated mitogenicity.

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 focus 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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[2] J. Lennartsson, E. Rollman, and L. Rönnstrand, unpublished observation.

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