**Gab2 Is Involved in Differential Phosphoinositide 3-Kinase Signaling by Two Splice Forms of c-Kit**

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The stem cell factor receptor/c-Kit plays an important physiological role in hematopoiesis, melanogenesis, and gametogenesis. It has also been implicated in numerous human malignancies. Signal transduction pathways shown to be of importance for c-Kit-mediated transformation include the phosphoinositide 3-kinase (PI3K)/Akt pathway. We have previously shown for c-Kit-mediated transformation include the phosphoinositide 3-kinase (PI3K)/Akt pathway. We have previously shown that two alternative splice forms of c-Kit, denoted GNNK\(^{-}\) and GNNK\(^{+}\), mediate distinctively different signals. In this study, we found that in the hematopoietic cell line Ba/F3, GNNK\(^{-}\) c-Kit mediates a substantially stronger activation of PI3K/Akt than GNNK\(^{+}\) c-Kit. This difference in signaling was shown to be dependent on the association of the scaffolding protein Gab2 with c-Kit, and Src-mediated phosphorylation of Gab2 was shown to be to be independent of the direct association of PI3K with c-Kit. Furthermore, proliferation and survival of Ba/F3 cells expressing a mutant of c-Kit that fails to bind to PI3K directly were slightly decreased compared with wild-type c-Kit-expressing cells. Using small interfering RNA technology, we further verified a role of Gab2 in inducing activation of PI3K/Akt downstream of c-Kit. To summarize, we show that PI3K activation by c-Kit is both splice form-dependent and cell type-specific. Furthermore, activation of PI3K by c-Kit is dependent both on the direct PI3K-binding site in c-Kit and on the phosphorylation of Gab2. The fact that c-Kit has been found mutated in numerous human malignancies, including acute myeloid leukemia, and that Gab2 is often overexpressed in acute myeloid leukemia suggests a potential role of Gab2-mediated PI3K activation in transformation.

The receptor for stem cell factor (SCF),\(^{2}\) 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 (for review, see Ref. 1). Furthermore, the c-kit gene is identical to the white spotting locus (W) in the mouse. Loss-of-function mutations in c-Kit lead to defects in melanogenesis, gametogenesis, and hematopoiesis. Stimulation of the c-Kit receptor with its ligand, SCF, leads to dimerization of receptors and activation of its intrinsic tyrosine kinase activity. Specific tyrosine residues are autophosphorylated, which results in the activation of downstream signaling pathways, including both positive and negative pathways.

As a result of alternative mRNA splicing, four isoforms of c-Kit have been identified in humans and two in mice (2, 3). In both mice and humans, alternative splicing results in isoforms characterized by the presence or absence of a tetrapeptide sequence, GNNK, in the extracellular part of the juxtamembrane region. Variants GNNK\(^{-}\) and GNNK\(^{+}\) (also denoted KitA and KitB, respectively) are coexpressed in most tissues, with the GNNK\(^{-}\) form predominating (4). In NIH3T3 cells transfected with either isoform of c-Kit, it was demonstrated that upon ligand stimulation, the GNNK\(^{-}\) isoform was more strongly tyrosine-phosphorylated and more rapidly internalized. Furthermore, it activated Erk more strongly than the GNNK\(^{+}\) isoform, whereas the activation of phosphoinositide 3-kinase (PI3K) was at similar level in the two splice forms (5). However, because c-Kit is not normally expressed in fibroblasts, but rather plays an important role in hematopoietic cells, we chose to study how the two splice forms of c-Kit signal in the pro-B cell line Ba/F3 to determine the role of alternatively spliced c-Kit in a more physiological setting.

Activation of PI3K by c-Kit has been linked to mitogenesis, differentiation, survival, adhesion, secretion, and actin cytoskeletal reorganization. In c-Kit, Tyr\(^{721}\) has been found to directly interact with PI3K (6) and was initially claimed to be essential for PI3K activation (7). However, it was later shown that in addition to the direct recruitment of PI3K to c-Kit, also the indirect recruitment via Gab2 is important for full PI3K activation (8, 9). The scaffolding protein Gab2 is expressed in many tissues, including brain, kidney, lung heart, bone marrow, testis, and ovary, and has been found to be a positive mediator of cytokine and growth factor signaling (for review, see Ref. 10). It can be activated by c-Kit and was found to be required for mast cell development and function (8, 11). SCF-induced Erk and Akt activation is decreased in Gab2-deficient bone marrow-derived mast cells (8), which indicates a role of Gab2 in PI3K/Akt activation.

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**PI3K/Akt activation.** c-Kit promotes cell survival via several mechanisms. PI3K-dependent activation of Akt and phosphorylation of Bad, a pro-apoptotic molecule, at Ser\(^{136}\) in vivo lead to its binding to 14-3-3.
protein and sequestering from the anti-apoptotic molecule Bcl-xL. Akt has also been shown to phosphorylate Foxo transcription factors, leading to their exclusion from the nucleus and inability to act in pro-apoptotic signaling (12). Using immortalized murine progenitor cells transduced with the Y721F/D816V mutant of c-Kit, Chian et al. (13) showed that transformation by this constitutively active form of c-Kit is dependent on PI3K.

In this study, we investigated the mechanisms of splice form-specific signaling of c-Kit in a hematopoietic cell line, Ba/F3, and found that in contrast to our previous findings in fibroblasts (5), the GNNK isoform of c-Kit mediates a markedly stronger activation of PI3K than the GNNK+ isoform. We further demonstrate that this difference arises from differential activation of Src family kinases and subsequent phosphorylation of Gab2. By mutating the direct PI3K-binding site in c-Kit, we show that the splice form-specific effect is not mediated through differences in direct association between the two splice forms. We further demonstrate that binding and phosphorylation of Gab2 require interaction with c-Kit through indirect binding via the adapter protein Grb2 to Tyr703 and Tyr725 in c-Kit. Using small interfering RNA (siRNA) against Gab2, we show that knockdown of Gab2 expression leads to a significant reduction in PI3K activation. To summarize, we demonstrate splice form-specific differences in PI3K signaling that could have consequences for the mode of activation of PI3K by c-Kit in transformed cells and provide a target for future therapeutic intervention with c-Kit signaling in tumors.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Peptide**—Recombinant human SCF was purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel). The rabbit antiserum KitC1, recognizing the C-terminal tail of c-Kit, was purified as described (14). Antibodies against Gab2, Akt, and SHP2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p85α antibody was from United States Biological (Swampscott, MA). The antibody against p110α has been described elsewhere (15). Anti-phospho-Akt antibody was from Cell Signaling Technology (Danvers, MA). Anti-Grb2 and anti-Grb2 antibodies were from BD Biosciences. Anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology (Swampscott, MA). The antibody against p110α/H9251 and Reagents

**Cell Culture**—The virus packaging cell line EcoPack (Clontech) and c-Kit-expressing NIH3T3 cells (5) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin; Ba/F3 cells and 32D cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 ng/ml recombinant murine interleukin-3 (IL-3). To establish c-Kit-expressing Ba/F3 and 32D cell lines, EcoPack cells were transfected with wild-type and variant mutant c-Kit constructs on pMSCVpuro, respectively; supernatants were collected to infect Ba/F3 or 32D cells, followed by a 2-week selection in puromycin. Expression of c-Kit was confirmed by flow cytometry and immunoblotting. c-Kit-expressing Ba/F3 and 32D cells were grown in the same medium as Ba/F3 and 32D cells but supplemented with 1.2 μg/ml puromycin.

**Cell Stimulation, Immunoprecipitation, and Western Blotting**—Ba/F3 and 32D cells were starved of IL-3 and serum for 5 h and stimulated with 100 ng/ml SCF for the indicated times. NIH3T3 cells were serum-starved overnight. Cells were washed once in ice-cold phosphate-buffered saline, lysed, and processed for immunoprecipitation and Western blotting as described (5). Immunodetection was performed by enhanced chemiluminescence using the SuperSignal Dura reagent and a CCD camera (LAS3000, Fujifilm).

**Electroporation of siRNA**—Silencing of Gab2 in c-Kit-expressing Ba/F3 cells was achieved by electroporation (300 V, 1500 microfarads) in a Gene Pulser II (Bio-Rad) in the presence of 100 nm Gab2 siRNA. Cells were incubated in the Ba/F3 growth medium for 12 h, starved for 5 h, stimulated with SCF, and lysed.

**Cell Survival Assay**—Cells were washed three times, resuspended in Ba/F3 complete medium without IL-3, and then seeded in 6-well plates containing 100 ng/ml SCF. Cells were seeded with 10 ng/ml IL-3 or without any cytokine as a control. After a 48-h incubation, cells were stained using the annexin V-PE apoptosis detection kit. Living cells, apoptotic cells, and dead cells were quantified by flow cytometry.

**RESULTS**

The Alternative Splice Form GNNK− Mediates a Much Stronger Activation of PI3K than the GNNK+ Isoform in Ba/F3 Cells—In a previous study (5), we showed in transfected NIH3T3 cells that there was no quantitative difference in the phosphorylation of Tyr725 of c-Kit, the binding site for PI3K, or in the activation of PI3K between the two splice forms GNNK− and GNNK+, respectively. However, because c-Kit is normally not expressed in fibroblasts, we sought to study the role of the two splice forms in a more relevant cell type. For this purpose, we chose the pro-B cell line Ba/F3, which was retrovirally transduced with either splice form of c-Kit. Equal expression of c-Kit was verified by flow cytometry (Fig. 1). Cells were stimulated with SCF, followed by lysis and separation by SDS-PAGE and electrotransfer to Immobilon P. The filter was probed with antibody against phosphorylated Akt (Ser473). In parallel, c-Kit phosphorylation was slower and weaker in cells expressing the GNNK+ isoform (Fig. 2A). Similar kinetics of Akt phosphorylation were seen in the myeloid cell line 32D transduced with the GNNK− and GNNK+ isoforms,
respectively (Fig. 2B). Furthermore, co-immunoprecipitation between the p85 subunit of PI3K and c-Kit was stronger and showed faster kinetics in cells expressing the GNNK\textsuperscript{-}H11002 isoform compared with the GNNK\textsuperscript{-}H11001 isoform (Fig. 2D). This is in strong contrast to the kinetics of Akt phosphorylation in NIH3T3 fibroblasts transduced with the GNNK\textsuperscript{-} and GNNK\textsuperscript{+} isoforms, respectively, where both isoforms of c-Kit elicited a response to the same level, although with slightly different kinetics (Fig. 2C) (5). However, whereas the overall tyrosine phosphorylation and the phosphorylation of the Src-binding site in c-Kit (Tyr568) were higher in the GNNK\textsuperscript{-} isoform, the direct binding site (Tyr721) showed similar levels of phosphorylation in both isoforms (Fig. 2D). Thus, the kinetics and magnitude of phosphorylation of individual tyrosine residues in c-Kit follow the same pattern in Ba/F3 cells as in NIH3T3 cells (5).

**PI3K Activation Is Dependent on Both Direct and Indirect Association with c-Kit**—The p85 subunit of PI3K is known to associate with c-Kit through phosphorylated Tyr\textsuperscript{721}. To evaluate its importance in SCF-stimulated PI3K activation, we generated two mutants of c-Kit that block the direct binding of p85 to c-Kit; Y721F blocks any protein binding to phosphorylated Tyr\textsuperscript{721}, whereas M724A is more selective for PI3K (16). The Y721F and M724A mutants of c-Kit blocked binding of p85 to either isoform of c-Kit, as expected (Fig. 3). However, the M724A mutant of either splice form of c-Kit was able to mediate phosphorylation of Akt despite its inability to associate with PI3K (Fig. 4). This suggests that additional pathways, apart from the direct binding of PI3K to c-Kit, mediate PI3K activation.

**Direct Binding of PI3K to Tyr\textsuperscript{721} in c-Kit Is Required for Cell Proliferation but Not for Cell Survival**—To investigate the role of direct versus indirect recruitment of PI3K to c-Kit in the mitogenic and survival response to SCF stimulation, Ba/F3 cells expressing either wild-type c-Kit or the Met\textsuperscript{724} mutant of either splice form were incubated in the presence or absence of SCF in medium devoid of IL-3 and scored for proliferation by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after 48 h of incubation. All cell lines showed SCF-dependent proliferation, with the cell lines expressing the GNNK\textsuperscript{-}H11002 isoform of c-Kit growing much faster than cells expressing GNNK\textsuperscript{-}H11001 (Fig. 5A). Blocking the direct binding of PI3K by the M724A mutant led to a decreased growth rate.

Cell survival assays were performed on the same cell lines using an annexin V-PE apoptosis detection kit (Fig. 5B). The data showed that cells expressing the M724A mutant of c-Kit, despite its inability to bind PI3K, could still survive in the presence of SCF, although cell death was increased. These data clearly indicate that the two splice forms of c-Kit differ in their ability to mediate cell growth and survival and that the direct binding of PI3K to c-Kit contributes to cell growth, but is not absolutely required for cell survival.
c-Kit Activates the PI3K/Akt Pathway via Gab2—To assess how the two isoforms of c-Kit activate the PI3K/Akt pathway without direct binding to PI3K, Ba/F3 cells expressing wild-type c-Kit or the Met724 mutant of either isoform were stimulated with SCF, and cell lysates were prepared and subjected to immunoprecipitation with antibodies against the regulatory subunit p85 or the catalytic subunit p110 of PI3K and then probed with anti-phosphotyrosine antibody 4G10 to detect binding partners of PI3K other than c-Kit. One potential candidate is the scaffolding protein Gab2. The blotting result showed that both c-Kit and Gab2 could bind to p85 and p110 after SCF stimulation of c-Kit. Furthermore, the M724A mutation did not affect the binding between Gab2 and p85 or p110 (Fig. 6). This indicates that PI3K can be activated by c-Kit either through direct recruitment to c-Kit or through indirect binding via Gab2. To elucidate the role of Gab2 in SCF-stimulated PI3K/Akt activation, Gab2 siRNA was introduced into Ba/F3 cells expressing c-Kit/M724A to knock down the expression of Gab2. This led to the suppression of Akt phosphorylation (Fig. 7), suggesting again that PI3K activation by c-Kit is mediated both through the direct binding to c-Kit and through the indirect association with Gab2. Filters were also probed with antibodies against Gab1, but it was found to be negative (data not shown). This is in agreement with a previous report (17).

Gab2 Activation Requires Src Family Kinase Activity and Grb2—Reports on a number of receptor systems have suggested that phosphorylation of Gab2 could be indirect and mediated through activation of Src family kinases. To investigate this possibility, Ba/F3 cells expressing either isoform of c-Kit were stimulated with SCF, subjected to immunoprecipitation with antibody against p85 and p110 (Fig. 6). This indicated that PI3K can be activated by c-Kit either through direct recruitment to c-Kit or through indirect binding via Gab2. To elucidate the role of Gab2 in SCF-stimulated PI3K/Akt activation, Gab2 siRNA was introduced into Ba/F3 cells expressing c-Kit/M724A to knock down the expression of Gab2. This led to the suppression of Akt phosphorylation (Fig. 7), suggesting again that PI3K activation by c-Kit is mediated both through the direct binding to c-Kit and through the indirect association with Gab2.
Splice Form-specific Activation of PI3K by c-Kit

SCF

Kit-  M724A-  Kit+  M724A+

IB: pAkt

IB: Akt

TCL

FIGURE 4. Loss of direct binding of PI3K to c-Kit leads to a partial reduction in Akt activation in either splice form of c-Kit. Ba/F3 cells expressing two isoforms of c-Kit with or without a p85-binding mutation were starved for 5 h, stimulated, and lysed. Total cell lysates (TCL) were probed with anti-phospho-Akt (pAkt) and anti-Akt antibodies. IB, immunoblot.

Parallel experiment was performed probing for phospho-Akt (Fig. 9B). The combination of both the Grb2-binding mutant and the Src family kinase inhibitor blocked Gab2 phosphorylation as well as Akt phosphorylation. From this result, we hypothesize that Grb2 can bring Gab2 to c-Kit through the binding of Grb2 to Tyr703 and Tyr936 of c-Kit, which then is phosphorylated through the action of Src family kinases. Phosphorylated Gab2 recruits the p85-p110 complex and activates the PI3K/Akt signaling pathway.

DISCUSSION

The SCF receptor/c-Kit is known to undergo alternative splicing, leading to the existence of at least six different isoforms of c-Kit in mice and humans. Two of these splice forms, GNNK− and GNNK+, exist as a result of alternative splice acceptor site usage and lead to the absence or presence of a tetrapeptide sequence, GNNK, in the extracellular part of the juxtamembrane region. We and others have previously shown that the two splice forms differ dramatically in signaling downstream of c-Kit (5, 20, 21). Although both isoforms are reported to bind SCF with equal affinity, the GNNK− isoform responds to SCF with a rapid and strong activation of its intrinsic kinase activity as well as Src family kinase activity. In contrast, GNNK+ responses are slower and weaker, and this seems to be due to a lower degree of activation of Src family kinases (5). It is both a qualitative and quantitative difference in signaling between the two splice forms. Probing of the receptor isoforms with phospho-specific antibodies revealed considerably higher levels of Tyr568 phosphorylation in the GNNK− isoform, whereas phosphorylation of Tyr721 was at similar levels in both isoforms (Fig. 2D) (5). The mechanisms by which these differences in signaling arise are incompletely known. From the structural data obtained through X-ray crystallography of the extracellular domain of c-Kit, one region has been identified that is involved in stabilization of ligand-induced receptor dimers (22). It is possible, but remains to be shown, that the absence or presence of the GNNK sequence in this region influences dimer stability.

The initial studies on how the two alternative splice forms of c-Kit signal were all performed on transfected NIH3T3 cells. However, c-Kit is normally not expressed in fibroblasts, and some important signal transduction molecules might be missing in fibroblasts. In this work, we studied the signaling of the two alternative splice forms in the pro-B cell line Ba/F3. We have demonstrated that, in contrast to what is seen in transfected NIH3T3 cells (Fig. 2C) (5), phosphorylation of Akt downstream of c-Kit is considerably stronger in transfected NIH3T3 cells expressing the GNNK− isoform than in NIH3T3 cells expressing the GNNK+ isoform (Fig. 2D). This occurs despite the fact that the phosphorylation of the direct binding site for PI3K, Tyr721, is phosphorylated to a similar extent in cells expressing either isoform of c-Kit (Fig. 2D). We also found the same to be true for the myeloid cell line 32D transfected with either the GNNK− or GNNK+ isoform of c-Kit (Fig. 2D). This is in contrast to recent findings by Young et al. (21), who studied c-Kit signaling in the murine FDC-P1 early myeloid cell line and Myb-immortalized hematopoietic cells and found marginal differences in PI3K association with c-Kit as well as in Akt phosphorylation.

An early study on c-Kit-mediated PI3Ks indicated the crucial importance of phosphorylated Tyr721 for activation of PI3K (7). However, the experiments were performed many times on the activity of PI3K physically associated with c-Kit and not the total cellular activity of PI3K. In this study, we have shown that mutation of Tyr721 only partially reduces SCF-dependent Akt phosphorylation (Fig. 4) in both GNNK− and GNNK+ iso-
forms. PI3K is known to be important for survival and proliferation of cells in a number of settings (23). To evaluate the impact of direct PI3K association with c-Kit on proliferation and survival, we performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays on Ba/F3 cells transfected with either wild-type or M724A mutant c-Kit (which fails to bind PI3K). As shown in Fig. 5A, loss of direct binding of PI3K to either splice form of c-Kit had only a marginal effect on SCF-mediated cell proliferation. Scoring for SCF-induced survival of these cells under conditions of IL-3 starvation demonstrated a partial effect on cell survival in either splice form of c-Kit (Fig. 5B). In contrast, treatment with a pharmacological inhibitor of PI3K has been shown to efficiently induce apoptosis of c-Kit-expressing cells (24).

Given the importance of PI3K in c-Kit-mediated survival, alternative pathways for activation of PI3K are likely to exist. Recent studies have reported the importance of the scaffolding protein Gab2 in c-Kit signaling. Gab2 is a scaffolding protein containing a pleckstrin homology domain, proline-rich sequences, and several tyrosine residues that are phosphorylated upon growth factor or cytokine stimulation, leading to recruitment of downstream signal transduction molecules (for review, see Ref. 25). Using targeted deletion of the gab2 locus in mice, Nishida et al. (8) noted a decrease in mast cell numbers in Gab2-deficient mice. Furthermore, SCF-stimulated Erk phosphorylation was markedly reduced in bone marrow-derived mast cells from such mice, indicating its importance in c-Kit signaling. Gab2 has been shown to regulate SCF-stimulated mast cell proliferation via SHP2-dependent activation of the Rac/Jun N-terminal kinase (JNK) pathway (9). It was further demonstrated that mutation of Tyr719 (corresponding to Tyr721 in the human sequence), the direct binding site of PI3K, did not fully inhibit PI3K activation in bone marrow-derived mast cells, but that Gab2 also could contribute to PI3K activation. In this study, we have shown that in Ba/F3 cells, c-Kit-induced activation of PI3K is mediated to about equal extents by the direct binding to c-Kit through Tyr721 and by the Src-dependent phosphorylation of Gab2 (Figs. 4 and 9). This is also consistent with the data obtained by Yu et al. (9), who demonstrated a dramatic reduction in Gab2 phosphorylation in bone marrow-derived mast cells expressing the Tyr567 (corresponding to Tyr568 in the human sequence) mutant of c-Kit that fails to activate Src family kinases. Association between Gab2 and receptor tyrosine kinases has in most cases been shown to occur indirectly through the adapter protein Grb2. Gab2 contains proline-rich sequences to which Grb2 binds through its SH3 domains, and the SH2 domain of Grb2 associates with phosphorylated tyrosine residues on the receptor tyrosine kinase. We have recently demonstrated that Grb2 in living cells associates with c-Kit through phosphorylated Tyr567 and Tyr936 (19). Because binding of Grb2 to phosphorylated tyrosine residues in the receptor tyrosine kinase. We have recently demonstrated that Grb2 in living cells associates with c-Kit through phosphorylated Tyr567 and Tyr936 (19). Because binding of Grb2 to phosphorylated tyrosine residues in the receptor tyrosine kinase. We have recently demonstrated that Grb2 in living cells associates with c-Kit through phosphorylated Tyr567 and Tyr936 (19). Because binding of Grb2 to phosphorylated tyrosine residues in the receptor tyrosine kinase. We have recently demonstrated that Grb2 in living cells associates with c-Kit through phosphorylated Tyr567 and Tyr936 (19). Because binding of Grb2 to phosphorylated tyrosine residues in the receptor tyrosine kinase. We have recently demonstrated that Grb2 in living cells associates with c-Kit through phosphorylated Tyr567 and Tyr936 (19). Because binding of Grb2 to phosphorylated tyrosine residues in the receptor tyrosine kinase. We have recently demonstrated that Grb2 in living cells associates with c-Kit through phosphorylated Tyr567 and Tyr936 (19). Because binding of Grb2 to phosphorylated tyrosine residues in the receptor tyrosine kinase. We have recently demonstrated that Grb2 in living cells associates with c-Kit through phosphorylated Tyr567 and Tyr936 (19). Because binding of Grb2 to phosphorylated tyrosine residues in the receptor tyrosine kinase.
these two Grb2-binding sites. To verify that Gab2 is indeed important for SCF-stimulated Akt activation, we used siRNA to selectively knock down expression of Gab2 (Fig. 7). Knockdown of Gab2 expression in combination with the M724A mutant of c-Kit further decreased Akt phosphorylation in both splice forms of c-Kit.

To summarize, we have found that the two splice forms of c-Kit, GNNK\(^{-}\) and GNNK\(^{+}\), differ in a cell type-specific manner in their mode of activation of PI3K/Akt. In the pro-B cell line Ba/F3 transfected with c-Kit, PI3K activation is dependent on the direct binding site in c-Kit, Tyr\(^{721}\), the association of the scaffolding protein Gab2 with c-Kit through Grb2, and Src-dependent phosphorylation of Gab2, whereas in NIH3T3 cells, it is dependent solely on phosphorylated Tyr\(^{721}\). The physiological function of PI3K in c-Kit signaling has been studied in mice expressing the Y721F mutant of c-Kit, giving rise to a phenotype with either defective spermatogenesis or defects in both spermatogenesis and oogenesis (26, 27) without affecting other c-Kit functions. It is tempting to speculate that these phenotypes might not fully reflect the function of PI3K in c-Kit signaling, but may be the result of tissues not expressing high enough levels of Gab2 to rescue the Y721F phenotype. However, this remains to be shown. Other studies targeting various isoforms of PI3K in mice have demonstrated an importance of the p110\(^{\alpha}\) isoform of PI3K in mast cells. Expression of a catalytically compromised mutant of p110\(^{\alpha}\) in transgenic mice led to defective SCF-mediated in vitro proliferation, adhesion, and migration and to impaired allergen-IgE-induced degranulation and cytokine release (28). Future studies are aiming at investigating the role of splice form-specific activation of PI3K and other signal transduction molecules in other cell type, such as melanocytes. Given the recently demonstrated occurrence of activating mutations of c-Kit in a subtype of melanoma (29), the role of alternatively splice isoforms of c-Kit in such a setting is important to investigate and will be the basis of future projects.

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FIGURE 8. SCF stimulation of either isoform of c-Kit leads to complex formation between Gab2, SHP2, and p85, whereas Grb2 association is constitutive. Ba/F3 cells expressing either isoform of c-Kit were starved for 5 h and stimulated with SCF for the indicated times. Cell lysates were immunoprecipitated (IP) with antibody against Gab2. After SDS-PAGE and electrophoretic transfer, membranes were probed for phosphotyrosine, Gab2, SHP2, p85\(^{\alpha}\), and Grb2. IB, immunoblot.

FIGURE 9. SCF-stimulated phosphorylation of Gab2 and Akt phosphorylation require both intact Grb2-binding sites on c-Kit and Src family kinase activity. Ba/F3 cells expressing either splice form of c-Kit with or without the Grb2-binding mutation N705A/N938A were starved for 5 h and incubated with the Src-selective inhibitor SU6656 for 30 min, followed by SCF stimulation. A, cell lysates were immunoprecipitated (IP) with anti-Gab2 antibody. Following SDS-PAGE and electrotransfer, membranes were probed for phosphotyrosine and Gab2. B, total cell lysates (TCL) were separated by SDS-PAGE, electrotransferred to Immobilon P, and probed with anti-phospho-Akt (pAkt) antibody (Ser\(^{473}\)) to show activation of the PI3K/Akt pathway. IB, immunoblot.
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