Impaired Shc, Ras, and MAPK Activation but Normal Akt Activation in FL5.12 Cells Expressing an Insulin-like Growth Factor I Receptor Mutated at Tyrosines 1250 and 1251*

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The Y1250F/Y1251F mutant of the insulin-like growth factor I receptor (IGF-IR) has tyrosines 1250 and 1251 mutated to phenylalanines and is deficient in IGF-I-mediated suppression of apoptosis in FL5.12 lymphocytic cells. To address the mechanism of loss of function in this mutant we investigated signaling responses in FL5.12 cells overexpressing either a wild-type (WT) or Y1250F/Y1251F (mutant) IGF-IR. Cells expressing the mutant receptor were deficient in IGF-I-induced phosphorylation of the JNK pathway and had decreased ERK and p38 phosphorylation. IGF-I induced phosphorylation of Akt was comparable in WT and mutant expressing cells. The decreased activation of the mitogen-activated protein kinase (MAPK) pathways was accompanied by greatly decreased Ras activation in response to IGF-I. Although phosphorylation of Gab2 was similar in WT and mutant cell lines, phosphorylation of Shc on Tyr113 in response to IGF-I was decreased in cells expressing the mutant receptor, as was recruitment of Grb2 and Ship to Shc. However, phosphorylation of Shc on Tyr239, the Src phosphorylation site, was normal. A role for JNK in the survival of FL5.12 cells was supported by the observation that the JNK inhibitor SP600125 suppressed IGF-I-mediated protection from apoptosis. Altogether these data demonstrate that phosphorylation of Shc, and assembly of the Shc complex necessary for activation of Ras and the MAPK pathways are deficient in cells expressing the Y1250F/Y1251F mutant IGF-IR. This would explain the loss of IGF-I-mediated survival in FL5.12 cells expressing this mutant and may also explain why this mutant IGF-IR is deficient in functions associated with cellular transformation and cell migration in fibroblasts and epithelial tumor cells.

The insulin-like growth factor I receptor (IGF-IR)1 activated by IGF-I and IGF-II has an important role in development and in the growth and survival of normal cells (1). There is also considerable evidence to indicate that the IGF-IR promotes maintenance of the malignant phenotype (2–4; reviewed in Refs. 5–7). Increased expression of IGF-I, IGF-II, and the IGF-IR has been documented in many human malignancies (8, 9) and overexpression of the IGF-IR can confer cells with a transformed phenotype (10, 11). Fibroblasts derived from Igf1r knockout mice cannot be transformed by a series of oncogenes and transformation can be restored by re-expression of the igf1r (12). Inhibition of IGF-IR expression or signaling capacity by antibodies (13), triple helix formation (14), antisense strategies (15), or dominant negative mutants (16) results in induction of apoptosis, failure to grow in anchorage-independent conditions, as well inhibition of metastasis.

To define the signaling pathways activated by the IGF-IR in its anti-apoptotic, mitogenic, or transforming functions, mutants of the receptor have been analyzed in several cellular systems. These include fibroblasts (17, 18), IL-3-dependent hematopoietic cell lines FL5.12 and 32D (6, 19), and tumor cell lines (20). These studies have identified domains in the C terminus of the IGF-IR that are associated with suppression of apoptosis, anchorage-independent growth, and the metastatic/invasive phenotype of tumor cells (6, 17). A double tyrosine to phenylalanine mutant Y1250F/Y1251F displays a very dramatic phenotype, in that cells expressing this mutant are completely deficient in suppression of apoptosis by IGF-I in response to IL-3 withdrawal, serum deprivation, or activation of c-Myc (6). The Y1250F/Y1251F mutant receptor is competent for mitogenic activity, but is also deficient in anchorage-independent growth (21), and cells expressing it have altered cytoskeletal organization (22) as well as decreased metastatic potential and synthesis of matrix metalloproteinases (20). However, the mechanism by which mutation of these two tyrosines results in specific abrogation of IGF-IR function has not been elucidated. There is no evidence of their phosphorylation by the IGF-IR (23) and proteins or pathways that interact with this site have not been identified.

Several signaling pathways have been associated with IGF-IR function. An activated IGF-IR recruits adapter proteins including IRS-1 or Shc (24, 25), Gab (26), and Crk (27) leading to activation of the MAPK (ERK) pathway, which has been associated with cell proliferation and differentiation (28); as well as the PI 3-kinase (phosphatidylinositol 3-kinase) pathway that leads to activation of Akt, Bad (29), and other downstream targets that suppress apoptosis and promote growth. A mitochondrial-associated pool of Raf-1 leading to Bad phosphorylation (30), and the stress kinase (SAPK/JNK) family, have also been linked with IGF-IR-mediated survival signaling. P38 activity is associated with rescue of cells from DNA damage (31), whereas both Akt and p38 activity and transient activation of
c-Jun-N-terminal kinase (JNK) in hematopoietic cells have been associated with protection from IL-3 withdrawal (32).

In this article, we investigated the signaling potential of the Y1250F/Y1251F mutant IGF-IR compared with the wild-type receptor. We found that this mutant is deficient in IGF-I-mediated activation of the MAPK pathways including ERK, JNK, and p38. This was accompanied by greatly diminished activity of the MAPKs. Western Blotting and Immunoprecipitation—FL5.12 cells overexpressing WT and mutant forms of the IGF-IR were seeded at 4 × 10^5 cells/ml in IMDM containing complete medium and cultured for 24 h, washed three times in serum-free medium, and then starved for 3.5 h in serum-free medium. Following serum starvation, cells were stimulated with IGF-I (100 ng/ml) for various times, and then washed in cold phosphate-buffered saline. Cells were lysed in ice-cold SDS lysis buffer (1% Nonidet P-40, 10 mM Tris, 50 mM NaCl, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.1% SDS, 1 µM peptatin, 1 mM phenylmethylsulfonyl fluoride, 1 µM aprotinin, and 1 mM sodium orthovanadate, pH 7.6). Debris was removed by centrifugation (20,800 × g) for 15 min at 4 °C, and samples were denatured by boiling in 5× Laemmli sample buffer for 5 min. Proteins were resolved by 4–15% gradient SDS-PAGE and blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Blots were blocked in TBS-T (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) with 5% milk for 30 min at room temperature. Primary antibodies were typically diluted 1:1000 in TBST, 5% goat serum or TBST, 5% milk and incubated overnight at 4 °C. Horseradish peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark) were used for detection by chemiluminescence with ECL reagent (Amersham Biosciences) or SuperSignal reagent (Pierce).

For immunoprecipitations cells were stimulated as above and lysed in ice-cold lysis buffer that did not contain SDS (i.e. 1% Nonidet P-40, 10 mM Tris, 50 mM NaCl, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, protease inhibitors, and 1 mM sodium orthovanadate) for 15 min. Nuclei and cell debris were then removed by centrifugation at 20,800 × g for 15 min at 4 °C. Following protein extraction, lysates were vortexed for 10 s and 200 µg of protein per sample were incubated with 5 µg of rabbit anti-Shc antibody or 5 µg of anti-Gab2 antibody overnight at 4 °C, followed by addition of 20 µl of bovine serum albumin-coated protein G-agarose beads for 3 h at 4 °C. Samples were rocked gently during this procedure. Immunoprecipitates were washed three times with ice-cold lysis buffer by centrifugation at 3000 rpm for 3 min. The beads were then boiled in 15 µl of 2X concentrated Laemmli sample buffer and proteins released into the sample buffer were separated by SDS-PAGE. Protein phosphorylation and proteins co-immunoprecipitating with Shc were identified using Western blotting as described above.

Ras Activation Assay—The Ras Activation Assay kit from Upstate Biotechnology was used to measure IGF-I-mediated Ras activation. In this system, cells are cultured in complete medium, starved, and stimulated with IGF-I as described above. Cells were washed in cold phosphate-buffered saline, and lysed in the supplied lysis buffer (25 mM Heps, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl2, 1 mM EDTA, and 10% glycerol) to which 1 µM pepstatin, 1 mM phenylmethylsulfonyl fluoride, 1 µM aprotinin, and 1 mM sodium orthovanadate were added. Active Ras was precipitated from the lysates using Ras Assay Reagent (a glutathione S-transferase fusion protein, corresponding to the human Ras binding domain of Raf-1, bound to glutathione-agarose beads), which specifically binds to and precipitates active Ras-GTP. Lysates and beads were incubated for 45 min at 4 °C with gentle agitation. Beads were washed three times with ice-cold lysis buffer, resuspended and boiled in 2 X Laemmli sample buffer. Western blotting with anti-Ras was used for detection of bead-bound activated Ras. Positive and negative controls were prepared by in vitro labeling of samples with either GTPγS or GDP, respectively, prior to affinity precipitation.
We have previously demonstrated that JNK, ERK, and Akt are phosphorylated in response to IGF-I stimulation in FL5.12/WT cells (32). To investigate differences in cells expressing the Y1250F/Y1251F mutant receptor we explored the ability of FL5.12/WT cells or FL5.12/Y1250F/Y1251F cells to activate these signaling pathways in response to stimulation with IGF-I. The PI 3-kinase pathway was activated to similar levels in both cell lines as indicated by Akt phosphorylation (Fig. 1C). Transient phosphorylation of the JNK pathway was diminished in FL5.12/Y1250F/Y1251F cells compared with FL5.12/WT cells. In addition activation of the ERK pathway was greatly reduced in Y1250F/Y1251F-expressing cells relative to FL5.12/WT cells as shown by phosphorylation of ERK (Fig. 1D). We had previously observed constitutive phosphorylation of p38 in these cells with or without IGF-I. Here we observed that transient phosphorylation of p38 in response to IGF-I stimulation for up to 5 min is deficient in cells expressing the mutant (Fig. 1E). Together, these data indicate that mutation of tyrosines 1250 and 1251 results in reduced JNK, ERK, and p38 activation, whereas Akt activation is not affected. This suggests that decreased activity of the MAPK families contributes to the loss of anti-apoptotic activity seen in cells expressing the Y1250F/Y1251F mutant receptor.

**MKK4 and c-Jun Activation Is Inhibited in the Y1250F/Y1251F Mutant Expressing Cells**

Although activation of JNK in response to IGF-I has previously been demonstrated in FL5.12 cells and other cells, very little is known about the contributions of the JNK pathway to IGF-I-mediated survival signaling or other functions. Because our data in Fig. 1 demonstrate that the Y1250F/Y1251F mutant showed decreased JNK activation in FL5.12 cells we further investigated the activation status of other members of the Jun kinase-signaling pathway. Activation of Jun kinases is thought to occur in a hierarchy of phosphorylation steps via a cascade of MAPKs that may assemble in a complex or scaffold (33). MAPK kinase-4 (MKK4) lies directly upstream from JNK. Fig. 2 shows that phosphorylation of MKK4 in response to IGF-I was diminished in FL5.12/Y1250F/Y1251F cells compared with FL5.12/WT cells. In addition, phosphorylation of the JNK substrate c-Jun was decreased in these cells. This indicates that activation of both an upstream and a downstream component of
the JNK pathway is deficient in cells expressing the Y1250F/Y1251F mutant IGF-IR. Altogether the data indicate that activation of the JNK pathway is blocked in cells expressing the Y1250F/Y1251F mutant. This together with the decreased activation of ERK and p38 suggests that either a scaffolding protein or other upstream signaling molecules necessary for activating the MAPK cascades are not active in cells expressing this mutant.

Ras Activation Is Decreased in FL5.12 Cells Expressing the Y1250F/Y1251F Mutant—Growth factor-mediated activation of the Ras superfamily of GTPases has been shown to contribute to activation of the MAPK and JNK pathways and contribute to growth, survival, transformation, and cytoskeletal organizations associated with cell movement (34, 35). Therefore, it was important to determine whether Ras activity was normal in FL5.12 cells expressing the Y1250F/Y1251F mutant. To do this we probed cell lysates from IGF-IR-stimulated cells for Ras protein or other upstream signaling molecules necessary for activating the MAPK cascades are not active in cells expressing this mutant.

Ras activation was measured by affinity precipitation of Ras from lysate samples. Activated Ras was eluted from the beads in 2 M Laemmli buffer and detected by Western blotting with anti-Ras antibody. Positive controls were generated by in vitro labeling of lysate samples from Y1250F/Y1251F cells with either GTP-$\gamma$S or GDP, respectively, prior to affinity precipitation. The lower panel shows Ras expression in the cell lysates.

Fig. 3. Ras activity is diminished in FL5.12/Y1250F/Y1251F cells. Cells were starved from IL-3 and FBS and stimulated with IGF-I for the indicated times. Agarose beads containing the Ras binding domain of Raf-1, which specifically binds to Ras-GTP, were used to affinity precipitate active Ras from lysate samples. Activated Ras was eluted from the beads in 2 M Laemmli buffer and detected by Western blotting with anti-Ras antibody. Positive controls were generated by in vitro labeling of lysate samples from Y1250F/Y1251F cells with either GTP-$\gamma$S or GDP, respectively, prior to affinity precipitation. The lower panel shows Ras expression in the cell lysates.

Deficient Shc, Ras, and MAPK Activity with Y1250F/Y1251F IGF-IR

Phosphorylation and recruitment of substrates to Shc is deficient in FL5.12/Y1250F/Y1251F cells, but Gab2 phosphorylation is unaffected. A, FL5.12/WT and FL5.12/Y1250F/Y1251F cells were seeded at 4 × 10^5/ml 24 h before harvest. Cells were then washed in serum-free medium, starved for 3.5 h, and stimulated with IGF-I (100 ng/ml) for 0, 5, or 10 min. Cells were lysed, and Gab2 was immunoprecipitated from the lysates as described under “Experimental Procedures.” Immunoprecipitated proteins were separated by SDS-PAGE, and Western blotting was performed with anti-phosphotyrosine and anti-Gab2. B, cells were starved as described above, and then stimulated with IGF-I for the indicated times. Following cell lysis, Shc was immunoprecipitated from the lysates, using polyclonal anti-Shc. Immunoprecipitated proteins were separated by SDS-PAGE, and Western blotting was performed with the antibodies indicated beside each panel. Blots were re-probed with monoclonal anti-Shc as a loading control.

Fig. 4. IGF-I-mediated Shc phosphorylation and recruitment of substrates to Shc is deficient in FL5.12/Y1250F/Y1251F cells, but Gab2 phosphorylation is unaffected. A, FL5.12/WT and FL5.12/Y1250F/Y1251F cells were seeded at 4 × 10^5/ml 24 h before harvest. Cells were then washed in serum-free medium, starved for 3.5 h, and stimulated with IGF-I (100 ng/ml) for the indicated times. Western blotting was performed using monoclonal anti-Shc and as antibodies directed to Grb2 phosphorylated at tyrosine 239 (P-Shc Y239) or at tyrosine 313 (P-Shc Y313).

Phosphorylation of substrates to Shc is deficient in FL5.12/Y1250F/Y1251F cells, but Gab2 phosphorylation is unaffected. A, FL5.12/WT and FL5.12/Y1250F/Y1251F cells were seeded at 4 × 10^5/ml 24 h before harvest. Cells were then washed in serum-free medium, starved for 3.5 h, and stimulated with IGF-I (100 ng/ml) for the indicated times. Western blotting was performed using monoclonal anti-Shc and as antibodies directed to Grb2 phosphorylated at tyrosine 239 (P-Shc Y239) or at tyrosine 313 (P-Shc Y313).

Phosphorylation of substrates to Shc is deficient in FL5.12/Y1250F/Y1251F cells, but Gab2 phosphorylation is unaffected. A, FL5.12/WT and FL5.12/Y1250F/Y1251F cells were seeded at 4 × 10^5/ml 24 h before harvest. Cells were then washed in serum-free medium, starved for 3.5 h, and stimulated with IGF-I (100 ng/ml) for the indicated times. Western blotting was performed using monoclonal anti-Shc and as antibodies directed to Grb2 phosphorylated at tyrosine 239 (P-Shc Y239) or at tyrosine 313 (P-Shc Y313).

Fig. 5. Shc phosphorylation at Tyr$^{313}$ but not at Tyr$^{239}$ is suppressed in FL5.12 cells expressing the Y1250F/Y1251F receptor. FL5.12/WT and FL5.12/Y1250F/Y1251F cells were seeded at 4 × 10^5/ml 24 h before harvest. Cells were then washed in IL-3 and serum-free medium, starved for 3.5 h, and stimulated with IGF-I (100 ng/ml) for the indicated times. Western blotting was performed using monoclonal anti-Shc as well as antibodies directed to Shc phosphorylated at tyrosine 239 (P-Shc Y239) or at tyrosine 313 (P-Shc Y313).
phosphorylation in FL5.12/WT and FL5.12/Y1250F/Y1251F cells, the phosphorylation status of specific sites on Shc were examined. Shc can be phosphorylated on three different tyrosine residues: Tyr239, Tyr240, and Tyr313 (equivalent to Tyr317 in human Shc). The Tyr239-Tyr240 sites are phosphorylated by Src (36) and have been associated with recruitment of Grb2. Tyr313 on Shc can bind an SH2 domain in Ship (37) and can also recruit Grb-2 (38). We investigated the phosphorylation status of these sites by using anti-phospho-Shc Tyr239 or anti-phospho-Shc Tyr313 antibodies. Data shown in Fig. 5 demonstrate that in IGF-I-stimulated FL5.12/Y1250F/Y1251F cells there was a decrease in the amount of Shc phosphorylated on Tyr313 compared with WT cells, whereas the cellular content of Shc phosphorylated on Tyr239 was equivalent in WT and mutant cells. This suggests that the decrease in Shc tyrosine phosphorylation observed in the total immunoprecipitated Shc (Fig. 4B) is because of decreased phosphorylation of Tyr313.

Overall, these data demonstrate that IGF-I-mediated phosphorylation of Shc is reduced on Tyr313 in cells expressing the Y1250F/Y1251F mutant and this is accompanied by decreased recruitment of both Grb2 and Ship. Lack of this tyrosine phosphorylation and recruitment of Grb2 and Ship may contribute to the reduced Ras and MAPK activation observed in these cells (35).

Inhibition of Src Activity Leads to Decreased Shc Phosphorylation on Tyr313, Decreased Ship Recruitment, and Differential Effects on MAPK Activation—The data above indicate that FL5.12 cells expressing the Y1250F/Y1251F mutant have decreased Shc phosphorylation and recruitment of Grb2 and Ship. To further investigate the role of Tyr313, and determine whether Ship has a role in IGF-IR signaling, we sought to inhibit Shc phosphorylation by blocking Src activity. It was also of interest to determine whether Src has any role in IGF-I-mediated Shc phosphorylation and activation of the MAPK pathways.

To do this, we used the Src inhibitor PP2. As can be seen in Fig. 6A, PP2 caused decreased IGF-I-mediated phosphorylation of Shc on Tyr313. Phosphorylation of Shc on Tyr313 was also slightly reduced. PP2 suppressed recruitment of Ship and only slightly affected Grb-2 recruitment to Shc in response to IGF-I (Fig. 6B). Interestingly, PP2 caused increased ERK activity and slightly increased Akt phosphorylation in FL5.12/WT cells, but did not alter IGF-I-mediated activation of JNK (Fig. 6C). PP2 also decreased p38 phosphorylation (Fig. 6D) and suppressed cell viability (not shown). This suggests that inhibition of Src activity, coupled with decreased phosphorylation of Shc at Tyr313, is sufficient to block activation of the ERK, Akt, or JNK pathways, but that Src-mediated phosphorylation of Shc and Ship may contribute to the activation of p38. This also suggests that Shc and Grb2 mediate activation of Ras, ERK, and JNK in these cells in a Src-independent manner.

The JNK Inhibitor SP600125 Inhibits IGF-I-mediated Survival and JNK Activation—We have previously shown that the ERK inhibitor, PD98059, did not have any effect on the viability of FL5.12 cells cultured in the presence of IGF-I or IL-3 (32), which suggests that ERK activity is not necessary for IGF-I-mediated survival in these cells. However, in that study we found that either PI 3-kinase inhibition or p38 inhibition was sufficient to suppress survival. We also showed that transient
activation of JNK and protection from apoptosis by the IGF-IR could be suppressed by dicumarol. To further investigate whether JNK is important for IGF-I-mediated anti-apoptotic signaling in these cells and whether it contributes to the loss of function of the Y1250F/1251F mutant we used the JNK inhibitor SP600125 (39). The effect of SP600125 on JNK activation by IGF-I in FL5.12/WT cells was assessed by Western blotting. Results shown in Fig. 7A demonstrate that IGF-I-mediated JNK phosphorylation was inhibited following 30 min preincubation with 30 μM inhibitor. Phosphorylation of Akt by IGF-I was retained in the presence of the inhibitor, which suggests that this survival pathway was maintained. IGF-I-mediated ERK phosphorylation was also unaffected; but basal levels of p38 phosphorylation were increased following preincubation with SP600125.

To test the effect of SP600125 on IGF-I-mediated cell survival, FL5.12/WT cells were cultured in the presence of FBS, IGF-I, or IL-3 for 48 h with increasing concentrations of SP600125 (5-30 μM). The results shown in Fig. 7B demonstrate that IGF-I-mediated suppression of apoptosis was inhibited in a concentration-dependent manner. At 5 μM SP600125 in the presence of IGF-I little decrease in cell viability was observed. Neither 5 nor 10 μM SP600125 had an effect on the viability of cells cultured in IL-3. In cultures supplemented with IGF-I and 20 μM SP600125 viability was decreased by ~45%, whereas 30 μM SP600125 decreased viability by ~70%. At 20 μM SP600125 cell viability in cultures supplemented with IL-3 was decreased by ~15% and at 30 μM viability was decreased by ~40%. Over 72 h of culture cell survival in the presence of IL-3 was not affected in the presence of 10 μM SP600125, whereas cell survival in the presence of IGF-I or 5% FBS was substantially inhibited (Fig. 7C).

Overall these data indicate that SP600125 inhibits JNK activation and inhibits IGF-I-mediated survival, whereas Akt, ERK, and p38 remain active. This is in agreement with previous results obtained with dicumarol (32) and JNK dominant negative constructs in T lymphocytes (40) and supports the conclusion that transient JNK activation contributes to IGF-I-mediated anti-apoptotic activity in FL5.12 cells.

**DISCUSSION**

Tyrosines 1250 and 1251 in the C terminus of the IGF-IR are required to promote suppression of apoptosis, cellular transformation (6, 17), control actin organization (18), and contribute to IGF-I-mediated tumor cell migration and invasion into tissues (20). The Y1250F/Y1251F mutant has been shown in several cellular systems to be deficient in these functions. However, the effects of this mutation on IGF-IR signaling or its mechanism of action have been difficult to elucidate. In this study we found, using the IL-3-dependent hematopoietic cell line FL5.12, that activation of the MAPK signaling pathways, activation of Ras, and the phosphorylation and assembly of the Shc signaling complex are all diminished in cells expressing the Y1250F/Y1251F mutant. Deficient activation of Ras-dependent signaling pathways could account for the lack of function of the Y1250F/Y1251F mutant in supporting cellular transformation, the cytoskeletal rearrangements associated with cell migration, and cell survival and differentiation in hematopoietic cells.

Although the Y1250F/Y1251F mutant has previously been analyzed for its signaling potential, a particular role for Shc and the Ras pathway has not been firmly established. In agreement with our observations in FL5.12 cells studies in 32D cells demonstrated that IGF-I-mediated phosphorylation of Shc proteins and co-immunoprecipitation of Grb2 with Shc was reduced (19) and this was associated with a decrease in the potential for cellular differentiation. Conversely, in NIH-3T3 cells replacement of tyrosines 1250 and 1251 of the IGF-IR (with phenylalanine and histidine, respectively) did not affect IGF-I-mediated phosphorylation of Shc or recruitment of Grb2 (22). Thus, there may be differences in the effects of this mutation on the phosphorylation of Shc in different kinds of cells. Alternatively, this may reflect different mechanisms of phosphorylation and function of Shc in hematopoietic cells and fibroblasts. This conclusion has been supported by an unpublished observation that transient transfection of the Y1250F/Y1251F mutant IGF-IR into IGF-IR null fibroblasts results in...
diminished IGF-I-mediated Shc phosphorylation, but normal IRS-2 phosphorylation and activation of Akt, ERK, and JNK. This suggests that IRS-2 can compensate for Shc in the activation of these signaling pathways in fibroblasts, but cannot in hematopoietic cells where IRS proteins are much less abundant than Shc. However, the functions of Shc in fibroblasts or epithelial cells are not yet fully elucidated.

Shc has been shown to play a role in a transformation by the polyoma middle T antigen and BCR-ABL (41, 42), and hyper-phosphorylation of Shc is seen in many different tumors (43). Here we found that IGF-I-mediated Shc phosphorylation was decreased on Tyr239 (Tyr237 in human Shc) in FL5.12/Y1250F/Y1251F cells. Tyr313 is a site for Grb2 recruitment (44) and Ras/MAPK activation. Phosphorylation of Tyr239, which is a Src phosphorylation site (44), was almost normal in the mutant expressing cells. Interestingly, both Tyr239 and Tyr313 (Tyr317) can bind the Grb2-SH2 domain, although some reports suggest that tyrosine 317 is more important for Grb2 binding (38). Because Grb2 recruitment to Shc was also decreased in FL5.12/Y1250F/Y1251F cells this suggests that Tyr313 is primarily responsible for Grb2 recruitment to Shc in these cells. This would also be consistent with decreased Ras activation (45) as well as the decreased MAPK activity.

Reduced Shc phosphorylation on Tyr313 in FL5.12/Y1250F/Y1251F cells was accompanied by decreased recruitment of the inositol phosphatase Ship, which cleaves the phosphate group from the 5' position of phosphatidylinositol 3,4,5-trisphosphate thus producing phosphatidylinositol 3,4-biphosphate (46). Ship has previously been shown to associate with Shc, and was proposed to have a competitive role in blocking the interaction of Shc with the Grb2-Sos complex of proteins that lead to Ras activation in B cells (47). Ship has also been shown to be necessary for tyrosine phosphorylation of Shc in response to stimulation with the antigen B cell receptor (48). In 3T3 L1 adipocytes Ship inhibits Glut 4 translocation and inhibits IGF-I-mediated membrane ruffling, indicating a role for this phosphatase in cytoskeletal rearrangement (49). Because the product of Ship is phosphatidylinositol 3,4-biphosphate, which can bind to several pleckstrin homology domain containing proteins including the RasGAP adapter protein p62 (dok) (50), it is also possible that Ship modulates or even amplifies signaling through Shc from the IGF-IR by interacting with pleckstrin homology proteins.

Decreased activation of the Ras pathway could explain the lack of function of the Y1250F/Y1251F mutant in cell survival as well as its lack of function in promoting clonalogenic growth and migratory capacity of transformed cells (6, 20–22). Ras has previously been shown to promote growth and migration through different signaling pathways in tumor cells (51) and to be an essential component of IGF-I and insulin signaling (52). Ras has also been implicated in the transformation activity associated with the IGF-IR. An IGF-IR truncated at the C terminus that is deficient in promoting anchorage-independent growth in IGF-I receptor null fibroblasts could be rescued from this phenotype by co-expression of Ras (53). There is some evidence that Ras-mediated transformation may require the involvement of JNK and p38 in addition to ERK. In a model of Ras-mediated transformation of rat epithelial cells it was noted that JNK activation promoted Ras transformation, whereas inhibition of p38 also facilitated JNK activation (54).

Ras has long been associated with direct activation of ERK through recruitment of Raf (55). More recently described Ras targets including the Rac and Rho G proteins, and an expanding series of guanine nucleotide exchange factors, including dbl, Vav, mSos, and the cyclic AMP-dependent nucleotide exchange protein Epac, have been shown to directly activate upstream JNK kinases leading to JNK activation (35, 56, 57). We analyzed FL5.12 cells for activity of Rac and Vav, and although these proteins are detected in Y1250F/Y1251F expressing cells the levels of IGF-1-mediated activation observed were too low to conclusively establish their role in the JNK activation pathway.

The role of JNK in cell survival and transformation is controversial, and JNK has been implicated in both pro- and anti-apoptotic signaling (for review see Ref. 58). In transformed B-lymphocytes there is compelling evidence to indicate that JNK promotes survival and is also necessary for transformation by BCR-ABL (59). Survival signals from JNK can use a JunD pathway that collaborates with NF-xB to increase anti-apoptotic gene expression (60). Our data in FL5.12 cells, where the JNK inhibitor SP600125 suppressed the survival of cells cultured in IGF-I or in 5% FBS, support a role for JNK as a mediator of IGF-I-mediated survival signaling. In addition, the lack of JNK activation in FL5.12/Y1250F/Y1251F cells that are deficient in IGF-I-mediated survival suggests that reduced JNK activation contributes to loss of survival.

It was previously observed that activation of MAPKs including ERK and JNK by IGF-I in MCF-7 cells was enhanced by overexpression of the regulatory scaffolding protein RACK1, which interacts with the IGF-IR, integrins, Src, and other signaling proteins (61, 62). We investigated the possibility that RACK1 might regulate ERK and JNK activation in FL5.12 cells and the possibility that lack of RACK1 could cause the signaling defects in cells expressing the Y1250F/Y1251F mutant. However, we found that RACK1 does not interact with the IGF-IR in FL5.12 cells (not shown). This indicates that RACK1 does not regulate IGF-IR signaling in hematopoietic cells and that RACK1 function is not involved in activation of the MAPKs or in the phenotype of the Y1250F/Y1251F mutant in these cells.

An alternative possibility for the altered signaling from the Y1250F/Y1251F mutant is that this receptor may have defective endocytosis that could cause impaired Shc phosphorylation and MAPK activation. Inhibition of clathrin-mediated endocytosis can alter signaling responses from the epidermal growth factor and Trk receptors and also selectively suppress activation of MAPK, but not Akt (63, 64). Since the Y1250F/Y1251F motif is a double tyrosine like the Tyr674-Tyr675 motif in the JunD pathway that collaborates with NF-xB (59). Survival signals from JNK can use a JunD pathway that collaborates with NF-xB to increase anti-apoptotic gene expression (60). Our data in FL5.12 cells, where the JNK inhibitor SP600125 suppressed the survival of cells cultured in IGF-I or in 5% FBS, support a role for JNK as a mediator of IGF-I-mediated survival signaling. In addition, the lack of JNK activation in FL5.12/Y1250F/Y1251F cells that are deficient in IGF-I-mediated survival suggests that reduced JNK activation contributes to loss of survival.

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In summary we have shown that phosphorylation of Shc, recruitment of Shc substrates, and activation of Ras plus the MAPK family members ERK, JNK, and p38 are deficient in cells expressing the Y1250F/Y1251F mutant IGF-IR. It may be important to further explore the mechanism and role of Shc phosphorylation as well as Ras pathway activity in IGF-I-mediated signaling in tumor cells.

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