Identification of Tyr-703 and Tyr-936 as the primary association sites for Grb2 and Grb7 in the c-Kit/stem cell factor receptor

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In this paper we demonstrate the presence of two novel *in vivo* autophosphorylation sites in the c-Kit/stem cell factor receptor (c-Kit/SCFR): Tyr-703 in the kinase insert and Tyr-936 in the C-terminal tail. We furthermore demonstrate that the adapter protein Grb2 is a specific binding partner for both phosphorylated Tyr-703 and phosphorylated Tyr-936, whereas the adapter protein Grb7 binds selectively to phosphorylated Tyr-936. It is

shown that the association occurs through the Src homology 2 (SH2) domains of Grb2 and Grb7. Binding of Grb2 to Tyr-703 in the c-Kit/SCFR provides a link to the Ras/mitogen-activated protein kinase pathway.

Key words: adapter protein, autophosphorylation, Ras/MAP kinase pathway, SH2 domain, tyrosine phosphorylation.

INTRODUCTION

The c-Kit/stem cell factor receptor (c-Kit/SCFR) is a transmembrane tyrosine kinase receptor encoded by the *c-kit* proto-oncogene which plays an important role in the normal development of haematopoietic cells, melanoblasts and germ cells (for a review, see [1]). Binding of its ligand, stem cell factor (SCF), leads to receptor dimerization and activation of its intrinsic tyrosine kinase activity. The subsequent autophosphorylation of specific receptor tyrosine residues creates specific recognition motifs for intracellular binding proteins that contain Src homology 2 (SH2) domains. SH2 domains are conserved stretches of about 100 amino acids which bind specifically to phosphotyrosine residues and are found in a variety of intracellular signalling molecules [2]. Thus it is of importance to identify the tyrosine residues that are autophosphorylated in order to understand the mechanisms by which c-Kit/SCFR signals. To date, limited information is available regarding the *in vivo* tyrosine phosphorylation sites on c-Kit/SCFR. Indirect data indicate that Tyr-721 becomes autophosphorylated upon ligand stimulation and binds phosphoinositide (phosphatidylinositol) 3-kinase [3,4]. Phosphorylated Tyr-568 and Tyr-570 have been shown to bind to members of the Src family of tyrosine kinases [3], to a mast cell tyrosine kinase [4] and to the protein tyrosine phosphatases SHP-1 and SHP-2 [5].

The adapter molecule Grb2 consists of one SH2 domain located between two SH3 domains, and has been shown to link receptor tyrosine kinases to the Ras/mitogen-activated protein kinase (MAP kinase) pathway. Grb2 occurs in a complex with the Ras GDP/GTP exchanger Sos, and the Grb2–Sos complex then binds to phosphorylated tyrosine residues [6], for example in the epidermal growth factor (EGF) receptor, insulin receptor substrate-1, Shc and the platelet-derived growth factor (PDGF) receptors [7–10]. The binding sites for Grb2 on these proteins have in common the presence of an asparagine residue in position +2 C-terminal to the phosphotyrosine residue. Association of Grb2 with c-Kit/SCFR has been demonstrated, but the exact binding site on the receptor has not been determined [11].

Although association of Grb2 with c-Kit/SCFR provides a link to the Ras/MAP kinase pathway, there are several other alternative pathways for activation of the Ras/MAP kinase pathway. The adapter protein Shc has been shown to be tyrosine-phosphorylated following activation of c-Kit/SCFR, leading to the creation of a Grb2 docking site on Shc [12]. The adapter proteins Gab-1 and Gab-2 become tyrosine-phosphorylated and associate with Grb2 following activation of c-Kit/SCFR [13]. Apart from its role in mediating activation of the Ras/MAP kinase pathway, Grb2 can also serve to bring the adapter molecule c-Cbl to c-Kit/SCFR [14]. Molecules related to Grb2, such as Grap [15], could (through recruitment of mSos1) activate the Ras/MAP kinase pathway.

Grb7 was identified by expression cloning using the autophosphorylated C-terminus of the EGF receptor as a probe [16]. It possesses one SH2 domain and a region of similarity to the non-catalytic domain of GTPase-activating protein, including a pleckstrin homology domain as well as a proline-rich region, but has no known catalytic activity (for a review, see [17]). Grb7 is thus an adapter, but its precise function remains to be elucidated. Its high similarity to Mig-10, a protein found in *Caenorhabditis elegans* which is involved in the migration of neuronal cells during embryonic development, points to an important function for Grb7 in cellular signalling [18]. Recently, a novel splice variant of Grb7, Grb7V, was found to be overexpressed in invasive oesophageal carcinomas [19]. Expression of antisense Grb7 led to decreased Grb7 levels and suppressed the invasive phenotype exhibited by oesophageal carcinoma cells. Grb7 is furthermore co-amplified with the HER2/ErbB2 receptor in some breast cancer cell lines and tissues [20,21], as well as in primary gastric tumours [22], and is known to bind to tyrosine-phosphorylated HER2 [23], Ret [24] and the PDGF β -receptor [25], as well as to a number of signal transduction molecules, such as SHP-2 [26] and Shc [21].

In the present study we have identified two novel autophosphorylation sites on c-Kit/SCFR. Upon stimulation by SCF, *in vitro* as well as *in vivo*, Tyr-703 in the kinase insert region and Tyr-936 in the C-terminus become phosphorylated. Fur-

Abbreviations used: EGF, epidermal growth factor; GST, glutathione S-transferase; c-Kit/SCFR, c-Kit/stem cell factor receptor; MAP kinase, mitogen-activated protein kinase; PAE, porcine aortic endothelial; PDGF, platelet-derived growth factor; SCF, stem cell factor; SH2, Src homology 2; WGA, wheatgerm agglutinin.

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thermore, we present evidence that the adapter molecule Grb2 associates directly with activated c-Kit at both autophosphorylation sites, Tyr-703 and Tyr-936, whereas Grb7 binds only to phosphorylated Tyr-936.

MATERIALS AND METHODS

Cell culture

Porcine aortic endothelial (PAE) cells expressing either human c-Kit/SCFR [27] or human PDGF α - or β -receptors [28,29] were cultured in Ham's F12 medium supplemented with 10% (v/v) fetal calf serum.

Antibodies, antisera and glutathione S-transferase (GST) fusion proteins

The rabbit peptide antiserum KitC1 against the C-terminus of c-Kit/SCFR was affinity-purified as described [30]. Anti-GST serum was raised against recombinant GST. The anti-phosphotyrosine antibody PY20 and an antibody against Grb7 were from Transduction Laboratories (Lexington, KY, U.S.A.), and peroxidase-conjugated donkey anti-(rabbit IgG) was from Amersham. Anti-Grb2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The KQE antiserum was raised against a peptide corresponding to amino acids 693–704 of c-Kit/SCFR (KQEDHAEALYK), and the ISE antiserum was raised against amino acids 928–946 of c-Kit/SCFR (ISESTNHIYSNLANCSPNR). The GST fusion proteins containing the SH2 domains of Grb2 (GST–Grb2SH2) and Grb7 (GST–Grb7SH2) were kindly provided by Dr. J. Schlessinger (Department of Pharmacology, NYU Medical Center, New York, NY, U.S.A.) and Dr. B. Margolis (Department of Internal Medicine and Biochemistry, University of Michigan Medical School, Ann Arbor, MI, U.S.A.) respectively.

Peptide synthesis and purification

Peptide synthesis and purification were performed as described by Hansen et al. [31]. Peptides were stored under dry conditions at room temperature. Peptide stock solutions were kept in 20 mM Hepes, pH 7.4, at -20°C . The following tyrosine-phosphorylated peptides were synthesized: HAEALY(p)-KNLLHSC (pY703 peptide) and ASTNHIY(p)SNLANCS (pY936 peptide). The corresponding non-phosphorylated peptides (Y703 and Y936 peptides) were also synthesized.

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting was performed essentially as described by Blume-Jensen et al. [30].

In vitro association of autophosphorylated c-Kit/SCFR with GST fusion proteins

Serum-starved PAE cells expressing wild-type c-Kit/SCFR (PAE/Kit cells) were stimulated or not with 100 ng/ml SCF for 5 min at 37°C , rinsed with ice-cold PBS and lysed in buffer containing 1% Triton X-100, 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 500 μM Na_3VO_4 , 1% Trasyolol and 1 mM PMSF. The lysates were centrifuged for 20 min at 14000 g and 4°C , and then incubated for 2 h at 4°C with 0.5 μg of GST fusion protein immobilized on glutathione–Sephacrose (Pharmacia). For peptide inhibition experiments, the lysates were incubated with immobilized fusion protein at 4°C for 1 h, various concentrations of synthetic peptides were added, and the incubation was continued for another 2 h. After washing three

times with lysis buffer, the samples were subjected to SDS/PAGE (7% gels) and immunoblotting was performed at room temperature using anti-phosphotyrosine antibodies (PY20).

GST blotting

PAE cells, stably expressing the PDGF α -receptor, the PDGF β -receptor or c-Kit/SCFR, were stimulated and lysed as described above. The lysates were incubated with wheat-germ agglutinin (WGA)–Sephacrose 6MB (Pharmacia) to enrich the glycoproteins. The samples were subjected to SDS/PAGE (7% gels) and transferred on to an Immobilon-P membrane. The membrane was incubated overnight at 4°C in a blocking solution (20 mM Hepes, pH 7.5, 5% non-fat dry milk and 0.02% sodium azide) and then probed for 1 h at room temperature in the same buffer using 1 $\mu\text{g}/\text{ml}$ GST–Grb7SH2 fusion protein. The fusion protein was detected by incubation of the membrane with anti-GST serum (1:200 dilution), followed by incubation with peroxidase-conjugated anti-(rabbit IgG). The membrane was washed for 3×15 min with a buffer containing 10 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.1% Triton X-100 before detection by ECL[®] (Amersham).

In vivo [^{32}P]P_i labelling of cells

[^{32}P]P_i labelling of cells was performed essentially as described by Blume-Jensen et al. [32].

In vitro kinase assay

In vitro kinase reactions were performed essentially as described by Blume-Jensen et al. [32].

In situ trypsin digestion, immunoprecipitation of peptides, phosphoamino acid analysis and Edman degradation

In situ trypsin digestion of labelled proteins and immunoprecipitation of tryptic peptides were performed essentially as described by Blume-Jensen et al. [32], with the exception that the ISE and KQE antisera were used for immunoprecipitation of peptides. Peptides were eluted with 1% (v/v) diethylamine, pH 11.5. An aliquot of peptide was subjected to phosphoamino acid analysis, while the remaining peptide was subjected to Edman degradation, as described in [32].

RESULTS

Identification of Tyr-703 and Tyr-936 as autophosphorylation sites in c-Kit/SCFR *in vitro* and *in vivo*

Due to the presence of an asparagine residue at position +2 relative to the tyrosine residue, Tyr-703 and Tyr-936 in c-Kit/SCFR were identified as potential candidates for association with Grb2 [33]. In order to determine whether Tyr-703 and Tyr-936 in c-Kit/SCFR are autophosphorylation sites, PAE/Kit cells were incubated with [^{32}P]P_i and stimulated with SCF, followed by lysis and immunoprecipitation using a c-Kit/SCFR-specific antiserum. ^{32}P -labelled c-Kit/SCFR was separated by SDS/PAGE, transferred to a nitrocellulose filter and digested *in situ* with trypsin. The resulting tryptic phosphopeptides were immunoprecipitated with antisera that recognize tryptic peptides that include either Tyr-703 (KQE) or Tyr-936 (ISE). The immunoprecipitated phosphopeptides were subjected to peptide sequencing, and the radioactivity released in each cycle of Edman degradation was measured. An aliquot of each sample was also subjected to phosphoamino acid analysis.

The KQE-antiserum recognizes an 11-amino-acid tryptic fragment which comprises residues 694–704 of c-Kit/SCFR (Figure

Figure 1 Analysis of SCF-induced phosphorylation of Tyr-703 of c-Kit/SCFR

(A) c-Kit/SCFR was immunoprecipitated from *in vivo* [32 P] P_i -labelled SCF-stimulated PAE/Kit cells. The radiolabelled c-Kit/SCFR was separated on SDS/PAGE, followed by transfer to a nitrocellulose filter and digestion with trypsin *in situ*. The tryptic peptides were subjected to immunoprecipitation using the KQE-antiserum, which recognizes a peptide comprising amino acids 694–704 of c-Kit/SCFR. The immunoprecipitated peptide was subjected to phosphoamino acid analysis and amino acid sequencing. The amount of radioactivity released in each cycle of Edman degradation was quantified using a Fuji BAS 2000 Bio-imager. Arrows indicate the tryptic cleavage sites. (B) c-Kit/SCFR was immunoprecipitated from lysates of SCF-stimulated PAE/Kit cells, subjected to an *in vitro* phosphorylation reaction in the presence of [γ - 32 P]ATP, separated by SDS/PAGE, transferred to nitrocellulose and digested *in situ* with trypsin. Tryptic peptides were subjected to immunoprecipitation, phosphoamino acid analysis and amino acid sequencing, as above. The insets show phosphoamino acid analyses of the tryptic peptides; S, T and Y indicate the positions of phosphoserine, phosphothreonine and phosphotyrosine respectively.

1A). The large amount of radioactivity released in cycle 10 corresponds to Tyr-703. Phosphoamino acid analysis of the same phosphopeptide indicated the presence of phosphotyrosine, without any phosphorylation on serine or threonine residues (Figure 1A, inset). In order to investigate whether the phosphorylation of Tyr-703 following stimulation by SCF of c-Kit/SCFR was a direct effect of the c-Kit/SCFR, or whether other tyrosine kinases activated following SCF stimulation were responsible for the phosphorylation, *in vitro* phosphorylation reactions were performed on immunoprecipitated c-Kit/SCFR. The immunoprecipitated receptor was digested with trypsin and subjected to secondary immunoprecipitation as described above. The immunoprecipitated peptide was only phosphorylated on tyrosine residues, and the radioactivity peaked in cycle 10 of Edman degradation, thus suggesting that Tyr-703 is a true autophosphorylation site (Figure 1B).

Figure 2 Analysis of SCF-induced phosphorylation of Tyr-936 in the C-terminus of c-Kit/SCFR

(A) *In vivo* [32 P] P_i -labelled PAE/Kit cells were stimulated with SCF and lysed. c-Kit/SCFR was immunoprecipitated and processed as described in the legend to Figure 1, except that the ISE-antiserum was used, which recognizes a peptide comprising amino acids 927–948 of c-Kit/SCFR. Arrows indicate the tryptic cleavage sites. (B) *In vitro* phosphorylation of wild-type c-Kit/SCFR. c-Kit/SCFR was immunoprecipitated from cell extracts of SCF-stimulated cells, subjected to *in vitro* phosphorylation in the presence of [γ - 32 P]ATP and digested *in situ* with trypsin as described. Tryptic peptides were subjected to immunoprecipitation, phosphoamino ELISA acid analysis and amino acid sequencing, as in (A). The insets show phosphoamino acid analyses of the tryptic peptides; S, T and Y indicate the positions of phosphoserine, phosphothreonine and phosphotyrosine respectively.

The ISE-antiserum recognizes a 22-amino-acid tryptic fragment comprising residues 927–948 of c-Kit/SCFR. When *in vivo*-labelled phosphopeptide that was immunoprecipitated by the ISE-serum was subjected to Edman degradation, one major peak of radioactivity was seen in cycle 10, corresponding to Tyr-936 (Figure 2A). There was also a marked peak of radioactivity in cycle 17, corresponding to Ser-943. Phosphoamino acid analysis revealed the presence of phosphoserine and phosphotyrosine in the peptide. Thus it can be concluded that Tyr-936 is phosphorylated upon stimulation by SCF of c-Kit/SCFR. *In vitro* labelling of c-Kit/SCFR followed by trypsin digestion and immunoprecipitation with the ISE-serum, along with Edman degradation, showed that Tyr-936 is a true autophosphorylation site (Figure 2B). Only tyrosine phosphorylation was seen in the *in vitro*-labelled c-Kit/SCFR. To summarize, these data show that Tyr-703 and Tyr-936 are autophosphorylation sites of the c-Kit/SCFR both *in vivo* and *in vitro*.

The SH2 domain of Grb2 associates *in vitro* with phosphorylated Tyr-703 and Tyr-936 of the autophosphorylated c-Kit/SCFR

The sequences C-terminal to Tyr-703 and Tyr-936 of the c-Kit/SCFR both have an asparagine residue in position +2

Figure 3 The Grb2 SH2 domain associates *in vitro* with autophosphorylated c-Kit/SCFR

PAE/Kit cells were stimulated or not with SCF and lysed. The lysate was then incubated either with 1 μ g of GST–Grb2SH2 fusion protein immobilized on glutathione–Sepharose (Grb2SH2) or with WGA–Sepharose (WGA). After washing the beads in lysis buffer, bound material was desorbed by boiling in Laemmli sample buffer and subjected to SDS/PAGE, followed by immunoblotting with anti-phosphotyrosine antibodies.

Figure 4 *In vitro* association of the Grb2 SH2 domain and autophosphorylated c-Kit/SCFR can be specifically competed with synthetic peptides corresponding to sequences including either Tyr-703 or Tyr-936

PAE/Kit cells were stimulated or not with SCF, lysed and incubated with 0.5 μ g of GST–Grb2SH2 fusion protein immobilized on glutathione–Sepharose for 1 h. Various concentrations of non-phosphorylated (Y703, Y936) or tyrosine-phosphorylated (pY703, pY936) synthetic peptides containing Tyr-703 (upper panel) or Tyr-936 (lower panel) of c-Kit/SCFR were added to the mixture, and the incubation was continued for 2 h. The samples were washed, subjected to SDS/PAGE and analysed by immunoblotting using anti-phosphotyrosine antibodies.

(pYKNL and pYSNL respectively), which is a requirement for binding of the adapter protein Grb2, which has been shown previously to associate with activated c-Kit/SCFR [11]. In order to investigate whether Grb2 associates with phosphorylated Tyr-703 and Tyr-936 of c-Kit/SCFR, lysates from SCF-stimulated PAE/Kit cells were incubated with a GST fusion protein containing the SH2 domain of Grb2 immobilized to glutathione–Sepharose. Associated material was subjected to SDS/PAGE and analysed by immunoblotting using anti-phosphotyrosine antibodies (Figure 3). A strong association was observed between GST–Grb2SH2 and the ligand-stimulated c-Kit/SCFR. Lysates from PAE cells expressing PDGF-BB-stimulated PDGF β -receptors were used as a positive control for the association with the GST–Grb2SH2 fusion protein [10]; the association of Grb2

Figure 5 The Grb7 SH2 domain associates *in vitro* with autophosphorylated c-Kit/SCFR

PAE/Kit cells were stimulated or not with SCF and lysed. Then 90% of the lysates were incubated with 0.5 μ g of GST–Grb7SH2 fusion protein immobilized on glutathione–Sepharose. The precipitated material was subjected to SDS/PAGE and analysed by immunoblotting using anti-phosphotyrosine antibodies. The PDGF β -receptor was used as a positive control of the association between receptor and GST–Grb7SH2 fusion protein, while the PDGF α -receptor was used as a negative control. The remaining 10% of the lysates was incubated with WGA–Sepharose as a control for receptor activation.

with the PDGF β -receptor was considerably weaker than that with c-Kit/SCFR.

To determine the specificity of the binding of Grb2 to c-Kit/SCFR, we repeated the experiment described above and added peptides corresponding to the sequences surrounding Tyr-703 and Tyr-936 (which were either phosphorylated or non-phosphorylated) at different concentrations. Both phosphorylated peptides, i.e. peptide pY703 (Figure 4, upper panel) and peptide pY936 (Figure 4, lower panel), were able to compete with the association *in vitro* of the Grb2 SH2 domain and c-Kit/SCFR in a dose-dependent manner. In contrast, neither of the non-phosphorylated peptides was able to compete with c-Kit/SCFR for binding to Grb2. These data were confirmed by experiments in which association of the GST–Grb2SH2 fusion protein with immobilized peptides pY703 and pY936 was demonstrated (results not shown). It could be shown that the Grb2 SH2 domain binds specifically to phosphopeptides pY703 and pY936. Thus we conclude that Grb2 is able to associate with phosphorylated Tyr-703 and Tyr-936 of c-Kit/SCFR.

The SH2 domain of Grb7 associates *in vitro* with Tyr-936 of autophosphorylated c-Kit/SCFR

The SH2 domain of Grb7 has been reported to associate directly with the autophosphorylated EGF receptor, [21], the Ret/glia-derived neurotrophic factor receptor [24] and the PDGF α -receptor [25]. In the PDGF α -receptor, Grb7 binds to the same phosphorylated tyrosine residues as does Grb2. This suggests that the SH2 domain of Grb7 recognizes an amino acid sequence motif with very similar specificity as that of Grb2. Thus we examined whether the SH2 domain of Grb7 binds directly to c-Kit/SCFR, and to which site(s). Tyr-703 and Tyr-936 are potential association sites, since they are the only tyrosine residues in c-Kit/SCFR that are followed by an asparagine residue in position +2. PAE/Kit cells were stimulated or not with SCF, followed by lysis in Triton X-100 buffer. The lysates were incubated with the GST–Grb7SH2 fusion protein immobilized on glutathione–Sepharose. The associated proteins were separated on SDS/PAGE and analysed by immunoblotting using anti-phosphotyrosine antibodies. The PDGF β -receptor was used as a positive control for the association, and the PDGF α -receptor was used as a negative control [25]. A clear association of the Grb7 SH2 domain with the ligand-activated c-Kit/SCFR was seen (Figure 5), as well as with the ligand-activated PDGF

Figure 6 The Grb7 SH2 domain associates directly with autophosphorylated c-Kit/SCFR

PAE/Kit cells or PAE/β cells were stimulated or not with SCF or PDGF-BB respectively and lysed, followed by incubation with WGA–Sepharose to enrich glycoproteins. The associated material was subjected to SDS/PAGE followed by electrotransfer to an Immobilon-P membrane. The filter was blocked with BSA and incubated with GST–Grb7SH2 fusion protein, followed by incubation with anti-GST antibodies. The PDGF β-receptor (PDGFβR) was used as a positive control for the association between receptor and the GST–Grb7SH2 fusion protein.

Figure 7 The *in vitro* association of the Grb7 SH2 domain and autophosphorylated c-Kit/SCFR can be specifically competed with synthetic peptides corresponding to a sequence including phosphorylated Tyr-936

PAE/Kit cells were stimulated or not with SCF and lysed in Triton lysis buffer. The clarified lysate was incubated with 0.5 μg of GST–Grb7SH2 fusion protein immobilized on glutathione–Sepharose for 1 h. Various concentrations of non-phosphorylated or tyrosine-phosphorylated synthetic peptide containing Tyr-703 (Y703, pY703) or Tyr-936 (Y936, pY936) of the c-Kit receptor were added to the mixture, and the incubation was continued for 2 h. The samples were washed, subjected to SDS/PAGE and analysed by immunoblotting with anti-phosphotyrosine antibodies.

β-receptor. In contrast, no association was seen with the ligand-activated PDGF α-receptor.

It has been shown previously that Grb2 associates directly with c-Kit/SCFR [13]. In order to clarify whether the association between Grb7 and c-Kit/SCFR is also a direct interaction, lysates from PAE/Kit cells stimulated with SCF were incubated with WGA–agarose to bring down c-Kit/SCFR. The associated proteins were separated by SDS/PAGE, followed by transfer to Immobilon-P. The filter was incubated with GST–Grb7SH2 fusion protein, followed by probing of the filter with anti-GST antibodies. Thus it could be demonstrated that the association between c-Kit/SCFR and Grb7 is a direct interaction (Figure 6).

To examine whether Grb7 binds to Tyr-703 and/or Tyr-936, lysates from SCF-stimulated PAE/Kit cells were incubated with GST–Grb7SH2 fusion protein, and phosphorylated or non-

phosphorylated peptides Y703 and Y936 were added at different concentrations. After SDS/PAGE and electrotransfer, detection with anti-phosphotyrosine antibodies showed that the association between the fusion protein and c-Kit/SCFR could not be competed by phosphorylated peptide pY703 (Figure 7). Thus the SH2 domain of Grb7 is unable to associate with phosphorylated Tyr-703. In contrast, the association between c-Kit/SCFR and Grb7 could be competed dose-dependently by phosphorylated peptide pY936, but not by the non-phosphorylated peptide Y936, indicating that the SH2 domain of Grb7 binds to phosphorylated Tyr-936 in c-Kit/SCFR (Figure 7). These data were confirmed by experiments in which association of the GST–Grb7SH2 fusion protein with immobilized pY703 and pY936 peptides was demonstrated (results not shown). Thus we conclude that Grb7 is able to associate with phosphorylated Tyr-936 of the autophosphorylated c-Kit/SCFR, but does not associate with phosphorylated Tyr-703.

DISCUSSION

In the present study, we show that Tyr-703 in the kinase insert and Tyr-936 in the C-terminal tail of c-Kit/SCFR become autophosphorylated after stimulation by SCF. The adapter protein Grb2 binds directly to both Tyr-703 and Tyr-936 in c-Kit/SCFR following ligand stimulation. The sequences surrounding Tyr-703 and Tyr-936 fulfil the consensus requirements for binding of Grb2, i.e. an asparagine residue is present in position +2, and either a hydrophobic or a glutamine residue is at position +3 [34]. On the other hand, Grb7 binds directly only to Tyr-936 in the C-terminus of c-Kit/SCFR, and not to Tyr-703. The SH2 domain of Grb7 has a binding specificity similar to that of Grb2. However, there are examples of tyrosine residues only binding either Grb2 or Grb7, while other tyrosine residues bind both Grb2 and Grb7. Thus, in SHP-2, Tyr-542 binds Grb2, but not Grb7 [26], while in ErbB3 Tyr-1180 and Tyr-1243 bind Grb7, but not Grb2 [35]. A comparison of known Grb2 and Grb7 association sites does not reveal any obvious determinant that selects for binding of one or the other (results not shown).

Our data show that Tyr-703 and Tyr-936 of c-Kit/SCFR are phosphorylated *in vivo* after stimulation by ligand, as well as *in vitro*, indicating that they are true autophosphorylation sites. The fact that both Grb2 and Grb7 recognize a similar binding motif raises the question of whether they can compete for the same binding site. It could be shown that Grb7 does not bind Tyr-703 in c-Kit/SCFR, and thus does not compete for binding of Grb2 to this site. In contrast, both Grb7 and Grb2 bound strongly to Tyr-936 in c-Kit/SCFR. Peptide competition experiments demonstrated that the Grb2 SH2 domain has a stronger affinity for Tyr-936 than does the Grb7 SH2 domain, since the binding of the former can be interfered with at very low concentrations of competitive phosphopeptide, whereas efficient competitive inhibition of Grb7 SH2 domain binding required 10–20 times higher concentrations of phosphopeptides. However, in the situation *in vivo*, other factors, such as the expression levels of Grb2 and Grb7 and their possible involvement in other signalling complexes, will influence which molecule will have preference for this site. These findings are similar to what was found for the PDGF β-receptor, in which phosphorylated Tyr-716 also showed a stronger affinity for Grb2 than for Grb7 [25]. Both Grb2 and Grb7 bind Tyr-580 of the tyrosine phosphatase SHP-2 [26], and Grb7 probably also recognizes the Grb2 binding site at Tyr-317 in Shc [21]. The binding of several SH2-domain-containing molecules to a single phosphotyrosine residue has been demonstrated previously. In the Met receptor, Tyr-1349 is

a docking site for multiple SH2 domains, and in the PDGF β -receptor Nck and phosphoinositide 3-kinase both compete for binding to Tyr-751 [36], whereas phospholipase C- γ 1 and SHP-2 both bind to Tyr-1009 [37].

In conclusion, site-directed mutagenesis of Tyr-703 and Tyr-936 in c-Kit/SCFR to phenylalanine residues will provide further insight into the role of Grb2 and Grb7 in c-Kit/SCFR-mediated biological responses, such as mitogenesis, chemotaxis, survival and differentiation.

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