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Interaction with the Phosphotyrosine Binding Domain/Phosphotyrosine Interacting Domain of SHC Is Required for the Transforming Activity of the FLT4/VEGFR3 Receptor Tyrosine Kinase*

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The *FLT4* gene encodes two isoforms of a tyrosine kinase receptor, which belongs to the family of receptors for vascular endothelial growth factor. As the result of an alternative processing of primary mRNA transcripts, the long isoform differs from the short isoform by an additional stretch of 65 amino acid residues located at the C terminus and containing three tyrosine residues, Tyr^{1333}, Tyr^{1337}, and Tyr^{1363}. Only the long isoform is endowed with a transforming capacity in fibroblasts. We show that this activity is related to the capacity of the tyrosine 1337-containing sequence to interact with the phosphotyrosine binding domain of the SHC protein. This demonstrates that a functional property of this newly described domain includes relay of mitogenic signals. In addition, it shows that the same receptor can mediate different functions through the optional binding of the phosphotyrosine binding domain and that the alternative use of this domain is sufficient to direct the signal toward different pathways.

Growth factors play a crucial role in cell activation. A large number of growth factor receptors have tyrosine kinase activity and trigger complex biochemical events, which lead to cellular differentiation or growth (1, 2). After ligand binding, receptor tyrosine kinases (RTKs)[1] undergo autophosphorylation on tyrosine residues and promote the subsequent phosphorylation of cytosolic targets (1).

The FLT1, FLK1/KDR, and FLT4 receptors show a high degree of similarity. They constitute a RTK class characterized by an extracellular region composed of seven immunoglobulin-like domains (3, 4) and involved in angiogenesis and vasculogenesis (5–12). FLT1/VEGFR1 and FLK1/KDR/VEGFR2 are high affinity receptors for factors of the vascular endothelial growth factor (VEGF) family (7, 13–16). *Flk1* −/− mice exhibit major defects in hematopoiesis and angiogenesis, whereas *Flt1* −/− mice are mainly deficient in vasculogenesis (11, 12). This suggests that the two receptors possess specific functions, in all likelihood mediated by different intracellular signal transduction pathways (17) and/or time expression.

FLT4 is a third receptor for a ligand of the VEGF family, VEGF-C (18). Its expression in adult mice appears predominant in the endothelial cells of lymphatic vessels (10). The human *FLT4* locus encodes two isoforms. The long form, FLT4L, differs from the short form, FLT4S, by the addition of 65 amino acids in the C-terminal region (19, 20). Analysis of the FLT4 signal transduction pathway has demonstrated that SHC is a major substrate of the FLT4 kinase (20, 21). The human *SHC* gene encodes three isoforms of 46, 52, and 66 kDa, respectively (22), which are substrates for many protein kinases (22–29). The p46 and p52 SHC isoforms are apparently generated by differential use of translation initiation sites, while the p66 isoform is derived from an alternative splicing event (22). All SHC proteins contain a C-terminal SH2 domain and an N-terminal domain called phosphotyrosine binding (PTB) domain, or phosphotyrosine interacting domain, allowing a dual interaction with tyrosine-phosphorylated sequences (22, 30–32). The Tyr^{317} tyrosine residue, located in between the PTB and the SH2 domains, represents the major phosphorylation site of SHC proteins (33) and constitutes a binding site for the GRB2 protein, an adaptor molecule providing a link with the RAS pathway (34). The PTB domain has been recently characterized as a domain involved in the association of the insulin receptor substrate-1 and SHC substrates with various RTKs and polyoma middle T antigen (MTAg) (35–42). The recognition sequence of the PTB domain is specified by residues N-terminal of the tyrosine and especially the −3 and the −5 residues, which correspond respectively to an asparagine and to a hydrophobic residue (32, 39, 41–43).

FLT4L, but not FLT4S, is endowed with a transforming potential, indicating the functional importance of the additional 65 C-terminal residues (20, 44). Three tyrosine residues, Tyr^{1333}, Tyr^{1337}, and Tyr^{1363}, are present in these 65 amino acids and are conserved between species (4). The Tyr^{1337} residue is required for FLT4L-mediated transformation, and its mutation dramatically reduces SHC phosphorylation, suggesting a potential role for this protein in FLT4 transforming activity (20). In this report, we show that the transforming potential of FLT4L is mediated by its interaction with the PTB

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[1] The abbreviations used are: RTK, receptor tyrosine kinase; VEGF, vascular endothelial growth factor; PTB, phosphotyrosine binding domain; MTAg, middle T antigen; mAb, monoclonal antibody; NBS, newborn serum; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; HA, hemagglutinin; GST, glutathione *S*-transferase; SH2, Src homology 2.

domain of SHC. This is based on the following arguments: (i) the Tyr^{1337} on FLT4L constitutes a SHC binding site. It is contained within a F^{1332}-*X*-N^{1334}-*X*-*X*-Y^{1337} sequence matching a potential PTB binding motif (32, 39, 41–43); (ii) the corresponding phosphorylated peptide binds to SHC; (iii) GST-SHC PTB recombinant proteins interact with the wild type receptor but very little with the Tyr^{1337} mutant receptor; (iv) deletion of the PTB but not of the SH2 domain abrogates SHC capacity to interact with the Tyr(P)^{1337}-containing peptide. Accordingly, the mutagenesis of Asn^{1334} or Tyr^{1337} residues abrogates FLT4L transforming activity. These results demonstrate the direct interaction between the FLT4L receptor and the SHC PTB domain, and attest to the functional relevance of the PTB domain in RTKs transforming capacity.

MATERIALS AND METHODS

Growth Factors, Antibodies, and Cell Cultures—Recombinant human CSF1 was purchased from Chiron (Cetus, St-Quentin-Yvelines, France). Anti-phosphotyrosine monoclonal antibody (mAb) (4G10), anti-p85 subunit of phosphatidylinositol 3-kinase mAb, anti-murine CSF1R mAb, and rabbit polyclonal anti-SHC antibody were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-GRB2 and anti-SHC mAb were purchased from Transduction Laboratories (Lexington, KY). Anti-HA1 mAb was purchased from Boehringer Mannheim. A polyclonal anti-FLT4 antibody was obtained by immunization of rabbits with a GST-FLT4 fusion protein containing the last 120 amino acids of FLT4L. Infected Rat2 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% newborn serum (NBS). The retroviral infection protocol and the characterization of the infected populations have been described elsewhere (20).

Generation of FF4 Chimeric Molecules and Site-directed Mutagenesis—The construction of FF4 chimeric molecules has been described (44). For mutagenesis, we used the Transformer site-directed mutagenesis kit (Clontech Laboratories, CA). Briefly, a 360-base pair *FLT4* fragment encompassing the portion of cDNA coding for Tyr^{1333}, Tyr^{1337} and Tyr^{1363} tyrosine residues was cloned in a pUC18 vector and the Tyr → to Phe, Asn^{1334} → Ala, and Leu^{1340} → Ala mutations were introduced using appropriate modified primers. Mutated cDNA fragments were totally sequenced by the dideoxynucleotide method, using double-stranded templates and T7 sequencing kit (Pharmacia Biotech Inc.), and reintroduced into wild type FF4L cDNA cloned in a Bluescript vector (Stratagene, La Jolla, CA). Full-length mutated FF4L cDNAs were then cloned into the retroviral LXSN vector (45).

Immunoprecipitation and Western Immunoblotting—5×10^6 Rat2 cells were incubated overnight in Dulbecco's modified Eagle's medium containing 0.5% NBS. Cells, stimulated with 0.5 $\mu g \cdot ml^{-1}$ of human CSF1 for 5 min at 37 °C, were then rinsed with cold PBS and lysed in lysis buffer (50 mM Hepes, pH 7.0, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate). Cells lysates, clarified by centrifugation at 13,000 rpm for 15 min, were incubated for 60–120 min at 4 °C with specific antibodies and protein-A coupled Sepharose (Pharmacia). Immune complexes were washed three times with HNTG buffer (50 mM Hepes, pH 7.0, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM sodium orthovanadate), heated in sodium dodecyl sulfate (SDS) sample buffer, separated by polyacrylamide gel electrophoresis (PAGE), transferred onto Immobilon (Millipore, St-Quentin-Yvelines, France), and probed with the appropriate antibody. Detection was performed with an ECL kit (Amersham, Les Ulis, France).

In Vitro Mixing Experiments with GST Fusion Proteins—Bacterially expressed GST fusion proteins, produced as described previously (24), were recovered from clarified lysates using glutathione-Sepharose beads (Pharmacia). Lysates from CSF1-induced cells grown in 100-mm tissue culture plates were mixed with 5 μg of recombinant protein on glutathione beads and incubated for 1 h at 4 °C. Protein complexes were washed three times with HNTG buffer, resolved by SDS-PAGE, transferred onto Immobilon membranes, and immunoblotted with the appropriate antibody.

Peptide Binding Assays—Unphosphorylated and tyrosine phosphorylated peptides derived from the primary amino acid sequence of the cytoplasmic domain of FLT4 were synthesized as described previously (43).

For ^{35}S cell labeling, after 1 h in methionine-free Dulbecco's modified Eagle's medium containing 3% NBS, Rat2 cells were metabolically labeled for 3–4 h with [^{35}S]methionine (3.7 $mBq \cdot ml^{-1}$) in the same medium. After extensive washings, cells were lysed in appropriate buffer.

Peptides were coupled on Affi-Gel beads according to the protocol of the manufacturer (Bio-Rad). Coupled peptide beads were incubated with labeled or non-labeled Rat2 lysates for 2 h and precipitated proteins were resolved on a 10% SDS-PAGE. The gel was either treated with EN^{3}HANCE (Amersham Corp.) prior to drying and exposure to x-ray film (Kodak XAR), or the proteins were Western-blotted onto Immobilon and revealed with appropriate antibodies.

Assays of Transforming Capacity—The soft agar assay was performed as follows: approximately 1×10^5 cells (from Rat2 origin)/60-mm Petri dish were seeded into an upper layer containing MEM 0.36% agar (Difco) supplemented with 10% NBS with or without 0.5 $\mu g \cdot ml^{-1}$ recombinant human CSF1. The number of colonies was scored after 15 or 21 days and representative areas photographed.

SHC DNA Constructs and Expression Vector—Four oligonucleotides were designed to introduce an in-frame p46 SHC cDNA into the pECE/HAP vector (46). This plasmid already contains the Kozak consensus sequence for initiation of translation and a sequence coding for a 9-residue immunodominant peptide from influenza virus hemagglutinin HA1 inserted between a *Hin*dIII site (5′) and an *Eco*RI (3′) site. The oligonucleotide SHC1 (5′-TCTTGAATTCG*AAGTACT*CCGGACCCG-GGGTTTCCTAC-3′) corresponds to the SHC N-terminal coding region and possesses an *Eco*RI site (underlined) allowing an in-frame insertion in the *Eco*RI site of the vector and a *Sca*I site (italics) allowing for the PTB internal deletion described below. A polymerase chain reaction (PCR) was performed on the SHC cDNA with the SHC1 and SHC2 (5′-AAACTGCAGTGAGCCATCAAAGCCAGCCAT-3′) oligonucleotides, and the 400-base pair PCR product was digested with *Eco*RI/*Pst*I enzymes (an underlined *Pst*I site was introduced in the SHC2 oligonucleotide) and cloned into Bluescript plasmid for sequencing. The SHC3 (5′-TTTGACATGAAGCCCTTCGAAGAT-3′) and SHC4 (5′-ATAATC-TAGATCATC*CCATGG*ATCACAGTTTCCGCTCCACAGG-3′) oligonucleotides amplified a fragment corresponding to the last 300 base pairs of SHC cDNA (nucleotides underlined and in italics represent introduced *Xba*I and *Nco*I sites, respectively). This fragment was also cloned into Bluescript vector after a *Pst*I/*Xba*I digestion and sequenced. The complete p46 SHC cDNA was assembled by cloning both PCR fragments and a *Bsm*I/*Pst*I SHC fragment representing the internal region of the SHC cDNA into Bluescript vector. The full-length p46 SHC cDNA was recovered by an *Eco*RI/*Xba*I digestion and cloned into the pECE-HA vector. The deleted PTB and SH2 SHC cDNAs were obtained by internal deletions with enzymes present in the SHC1 and SHC4 oligonucleotides and in the SHC cDNA sequence (*Sca*I for dPTB and *Nco*I for dSH2). An *Pst*I/*Xba*I fragment containing the whole SH2-SHC cDNA sequence was subcloned into pUC18 plasmid for Arg^{401} → Leu mutagenesis according to the manufacturer's instructions (Clontech Laboratories).

RESULTS

FF4L but Not FF4S Is Able to Associate with SHC Proteins in Cells—In the absence of an available FLT4 ligand, chimeric FMS-FLT4 (designated FF4S and FF4L, respectively) molecules, responsive to CSF1 growth factor, allowed the study of the functional behavior of FLT4 receptors in fibroblast cells (44). The SHC proteins constitute major substrates of the FLT4 receptor in fibroblasts and endothelial cells (21, 44). Analysis of the interaction between FLT4 and SHC proteins has been restricted to the long form (21). We therefore undertook a comparative analysis of the association of the two isoforms with the SHC proteins. SHC proteins were immunoprecipitated from unstimulated or CSF1-stimulated Rat2 cells expressing FF4S or FF4L, resolved by electrophoresis, and analyzed by immunoblotting with anti-phosphotyrosine antibody (Fig. 1*A*). As shown previously (44), the p52 and p46 SHC proteins were highly phosphorylated after CSF1 stimulation in FF4L-expressing cells, whereas only a faint signal was observed for FF4S-expressing cells. No signal appeared with LXSN vector-infected cells. A phosphorylated 180-kDa protein, presumed to be the activated receptor, co-immunoprecipitated with the SHC proteins in stimulated FF4L-expressing cells (Fig. 1*A*); it comigrated with FF4L immunoprecipitated with anti-FLT4 antibody (Fig. 1*A*). The identity of this 180-kDa band with the activated FF4L receptor was confirmed by stripping and rep-

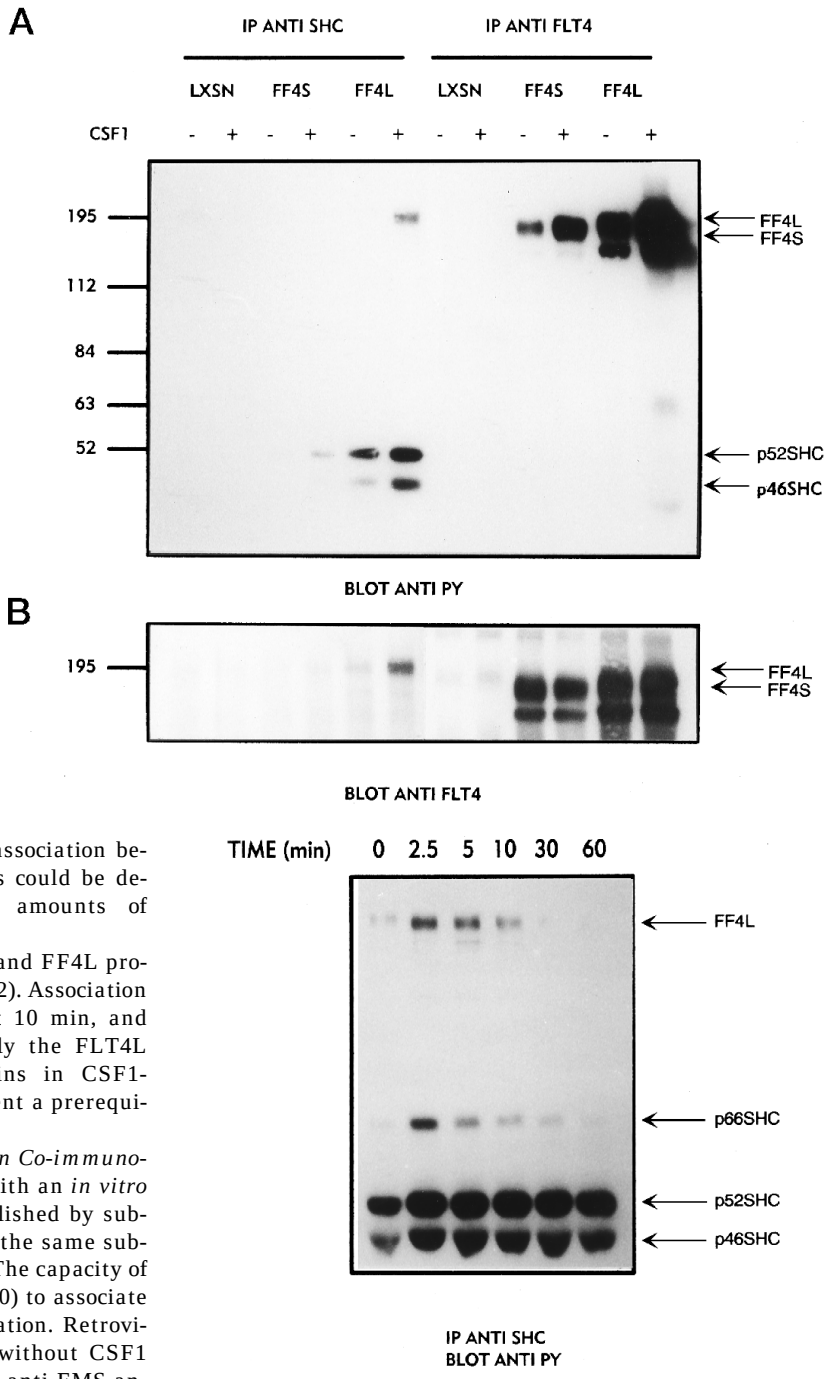

FIG. 1. **The SHC proteins associate with the FLT4L receptor.** Retroviral infected cell populations expressing no receptor (L*X*SN), FF4S, or FF4L chimeric molecules were starved overnight (0.5% NBS) and stimulated 5 min with medium containing no (−) or 0.5 $\mu g \cdot ml^{-1}$ CSF1 (+). After extensive washing, cells were lysed and proteins were immunoprecipitated with anti-SHC or anti-FLT4 antibodies. After migration on a 7.5% SDS-PAGE and transfer, the blot was sequentially probed with anti-phosphotyrosine (*anti-PY*, *A*) and anti-FLT4 antibody (*B*). *Arrows* indicate the identity of the phosphorylated bands corresponding to the receptors and to the SHC proteins isoforms. Constitutive phosphorylation of the receptors can be observed (as reported previously; Ref. 44) but does not induce SHC association.

robing with anti-FLT4 antibody (Fig. 1*B*). No association between the FF4S isoform and the SHC proteins could be detected, although the cells expressed high amounts of phosphorylated FF4S (Fig. 1).

The kinetics of association between the SHC and FF4L proteins after CSF1 stimulation was analyzed (Fig. 2). Association was easily detectable at 2.5 min, decreased at 10 min, and disappeared by 30 min. We conclude that only the FLT4L receptor can associate with the SHC proteins in CSF1-stimulated Rat2 cells; this property may represent a prerequisite for substrate phosphorylation.

Tyr^{1337} Mutation Abolishes SHC/FF4L Protein Co-immunoprecipitation—FF4L but not FF4S is endowed with an *in vitro* and *in vivo* transforming activity, which is abolished by substitution to phenylalanine of Tyr^{1337} but not by the same substitution of residues Tyr^{1333} or Tyr^{1363} (20, 44). The capacity of FLT4L receptors carrying these substitutions (20) to associate with SHC was investigated by immunoprecipitation. Retrovirus-transduced Rat2 cell populations, with or without CSF1 stimulation, were first immunoprecipitated with anti-FMS antibody, specific of the extracellular region of the chimeric molecule, then subjected to a sequential immunoblot analysis with anti-phosphotyrosine (Fig. 3*A*, *upper panel*) and anti-FLT4 antibodies (Fig. 3*A*, *lower panel*). As reported previously, both FF4S and the mutated FF4L.37F receptor present a weaker tyrosine phosphorylation after CSF1 stimulation (but not a weaker kinase activity), possibly because of the absence of the Tyr^{1337} autophosphorylation site (20). As expected, a phosphorylated 52-kDa protein was specifically co-immunoprecipitated with FF4L, FF4L.33F, and FF4L.63F but not with FF4S and FF4L.37F molecules (Fig. 3*A*, *upper panel*). We presume that this band represents the p52SHC protein, but we were unable to reveal it with either monoclonal or polyclonal anti-SHC antibodies.

In the reverse experiment, unstimulated and CSF1-stimulated Rat2 cell lysates were incubated with anti-SHC antibody; the precipitated proteins were analyzed by Western

FIG. 2. **Kinetics of association between SHC and FLT4.** FF4L-expressing cells were starved and stimulated by 0.5 $\mu g \cdot ml^{-1}$ CSF1 during various lengths of time. The lysates were immunoprecipitated with anti-SHC antibody. After migration and transfer, proteins were revealed with anti-phosphotyrosine (*anti-PY*) antibody. In this figure, the blot was overexposed to evidence the p66SHC protein (signals for p46 and p52 are thus saturated).

blotting with anti-phosphotyrosine antibody (Fig. 3*B*, *upper panel*). Four major phosphorylated bands of 180, 66, 52, and 46 kDa, corresponding, respectively, to the receptor and the different SHC isoforms, appeared in FF4L-expressing cells; a 120/130-kDa phosphorylated protein was also co-immunoprecipitated with the SHC proteins observed after longer exposures of the blot (data not shown). The mutated Tyr^{1333} (not shown) and Tyr^{1363} receptors retained the capacity to phosphorylate the SHC proteins and to associate with them after activation, whereas the Tyr^{1337} mutation dramatically diminished the phosphorylation of SHC proteins and their association with

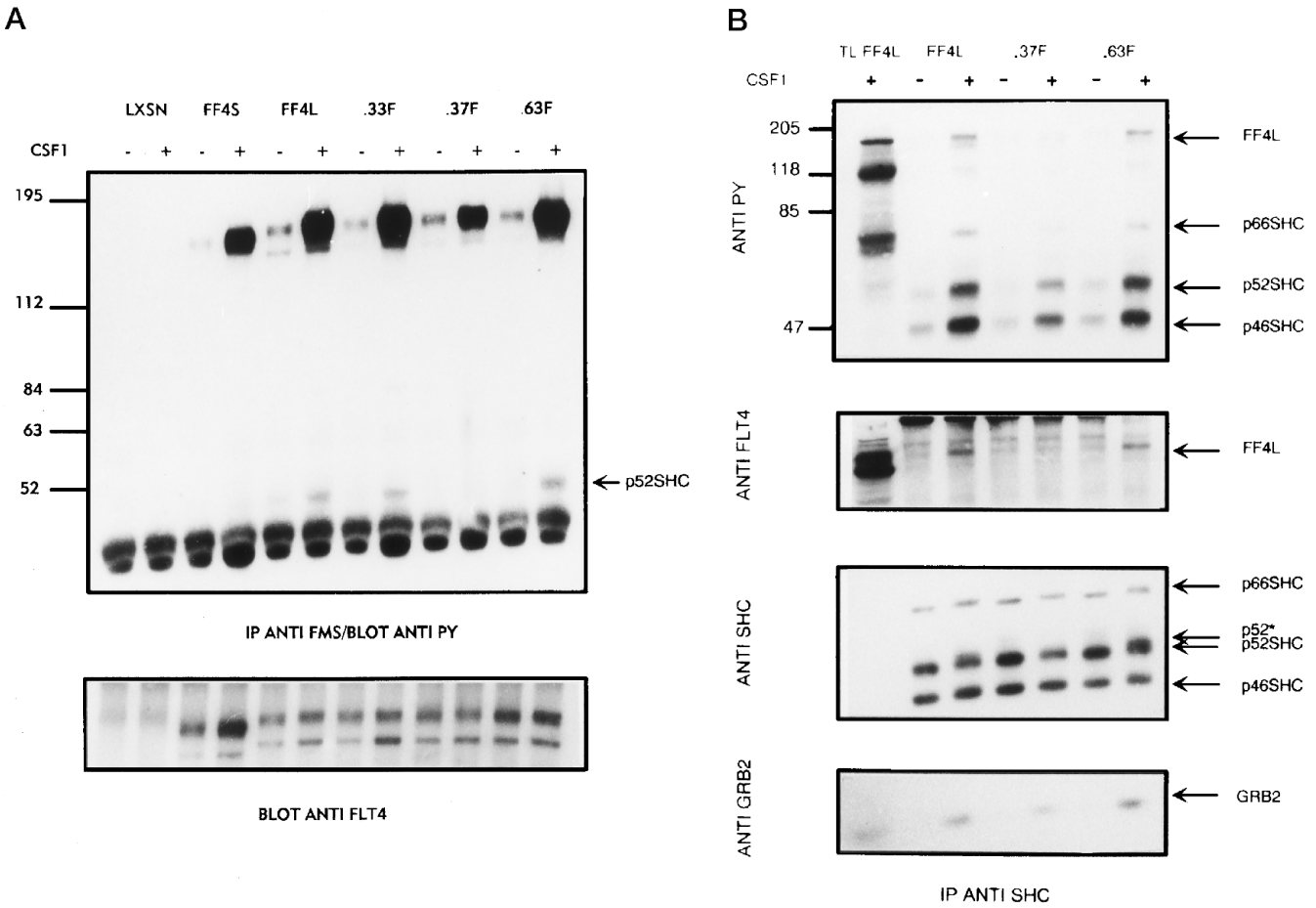

FIG. 3. **Mutation at tyrosine residue 1337 abolishes FLT4-SHC interaction.** *A*, Rat2 infected populations expressing either no receptor (L*X*SN), the wild type chimeric molecules (FF4S and FF4L), or the FF4L mutated receptors (.33F, .37F, and .63F) were CSF1-stimulated for 5 min. The respective lysates were incubated with anti-FMS antibody, and the precipitated proteins were transferred onto Immobilon and probed with anti-phosphotyrosine antibody (*A*, *upper panel*) or anti-FLT4 antibody (*A*, *lower panel*). The *arrow* indicates the putative phosphorylated p52SHC protein. *B*, the same experiment was performed except that the proteins were immunoprecipitated by anti-SHC antibody and migrated on a 10% SDS-PAGE. Transferred proteins were revealed with anti-phosphotyrosine (*upper panel*), anti-FLT4 (*second panel*), mAb anti-SHC (*third panel*), and anti-GRB2 (*lower panel*) antibodies. The first lane (*TL FF4L*) represents a CSF1-stimulated FF4L population total lysate. The SHC proteins are only visible on the first lane after a longer exposure (*third panel*, data not shown). *p52** indicates a p52SHC protein with a slower migration in SDS gel.

the receptor (Fig. 3*B*, *upper panel*). Identification of the 180-kDa phosphorylated band as FF4L and quantitation of the immunoprecipitated SHC proteins were obtained by additional sequential probings of the membrane with anti-FLT4 (Fig. 3*B*, *second panel*) and monoclonal anti-SHC (Fig. 3*B*, *third panel*) antibodies, respectively. A shift in the mobility of the highly phosphorylated p52SHC proteins (p52*) was observed in CSF1-stimulated FF4L- and FF4L.63F-expressing cells (see also Fig. 7*C*). Anti-GRB2 immunoblot demonstrated a 27-kDa protein in control cells and in CSF1-stimulated FF4L-, FF4L.33F-, and FF4L.63F-expressing cells (Fig. 3*B*, *lower panel*) but only a faint signal was detected in FF4L.37F cells, suggesting a direct relation between SHC phosphorylation and SHC/GRB2 association. These results were highly reproducible and altogether suggest that the Tyr^{1337} mutation abolishes the stable interaction between the SHC proteins and the activated FF4L receptor and decreases SHC phosphorylation and SHC/GRB2 association.

In Vitro Association between Tyr(P)^{1337}-containing Peptide and SHC Proteins—To document the direct interaction between the SHC proteins and the Tyr^{1337}-containing sequence, we designed differentially tyrosine phosphorylated peptides derived from the sequence encompassing this residue in FLT4L (Fig. 4*A*): nonphosphorylated peptide (peptide 1), $Tyr(P)^{1333}$ (peptide 2), $Tyr(P)^{1337}$ (peptide 3), and $Tyr(P)^{1333}/Tyr(P)^{1337}$ (peptide 4) phosphorylated peptides. Rat2 cell lysates were incubated with peptides conjugated to beads, and bound proteins were separated by SDS-PAGE. After protein transfer, the membrane was probed with anti-SHC antibody (Fig. 4*B*, *upper panel*) or control irrelevant antibody (anti-p85 antibody) (Fig. 4*B*, *lower panel*). Three proteins of p66, p52, and p46, coupled with peptides 3 and 4, were recognized by anti-SHC antibody, whereas, in the same conditions, anti-p85 detected only a faint band, attributed to a nonspecific binding to the peptide. The phosphorylated Tyr^{1337}-containing sequence therefore constitutes a binding site for SHC proteins *in vitro*.

The PTB Domain of SHC Interacts with FLT4L Tyr^{1337} Residue—The three related SHC proteins are characterized by the presence of SH2 and PTB domains (22). Previous reports have shown that the SHC SH2 domain binds to the FF4L receptor and to the Tyr^{1337} mutant, excluding the possibility that the sequence containing tyrosine 1337 forms a binding site for this domain (20, 21). To evidence the potential interaction between SHC PTB domain and FLT4L, a GST-PTB SHC protein was produced in *Escherichia coli* and the recombinant protein coupled to glutathione-Sepharose beads was used to isolate proteins from CSF1-stimulated Rat2 cells. Their immunoblotting with anti-phosphotyrosine antibody showed binding of GST-PTB SHC to the activated FF4L receptor. This binding was dramatically decreased with the Tyr^{1337} to F^{1337} mutation (Fig.

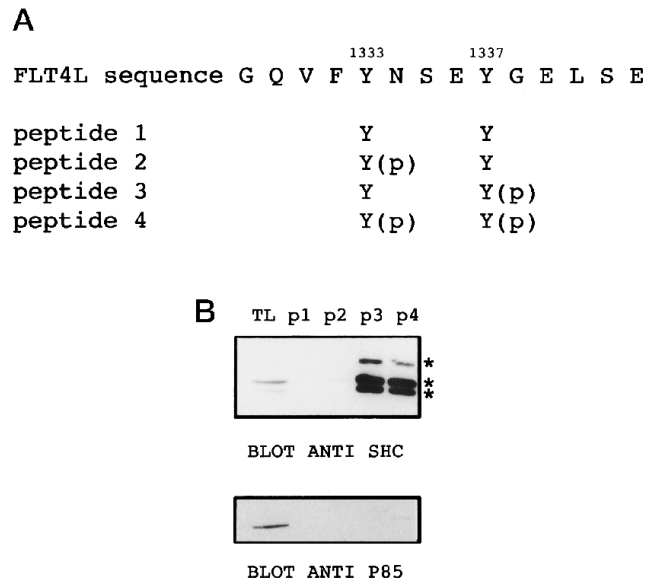

FIG. 4. **FLT4L derived-phosphorylated Tyr^{1337}-containing peptide binds to SHC proteins.** *A*, FLT4L specific sequence encompassing two of the three C-terminal tyrosine residues present in the long FLT4 form. Four peptides mimicking this sequence were designed. The tyrosine residues were either monophosphorylated (peptides 2 and 3), biphosphorylated (peptide 4), or unphosphorylated (peptide 1). *B*, Rat2 cell lysates were incubated with the different peptides (designated *p1–p4*) and bound proteins were electrophoresed on a 10% SDS-PAGE. After transfer, the membrane was probed with anti-SHC (*upper panel*) or, as control, anti-phosphatidylinositol 3′-phosphate (*PI3K*) p85 subunit (*lower panel*) antibody. The first lane (*TL*) represents a Rat2 cell total lysate.

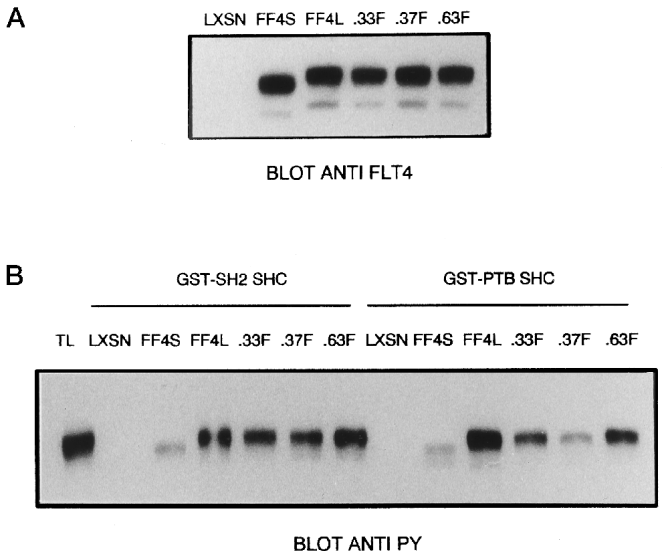

FIG. 5. **GST-PTB SHC interacts with FLT4L at Tyr^{1337}.** Infected Rat2 cell populations expressing the different chimeric molecules and their mutants were starved overnight in 0.5% NBS and stimulated with CSF1 (0.5 $\mu g \cdot ml^{-1}$) during 5 min. After lysis, an aliquot of each lysate was electrophoresed on a 7.5% SDS-PAGE and revealed with anti-FLT4 antibody (*panel A*). The remaining lysate was separated in two samples and incubated either with GST-SH2 SHC or with GST-PTB SHC recombinant proteins coupled to glutathione-Sepharose beads. Precipitated proteins were electrophoresed on a 7.5% SDS-PAGE and revealed with anti-phosphotyrosine antibody (*panel B*). The first lane (*TL*) represents a CSF1-stimulated FF4L population total lysate.

5*B*, *right panel*). It was also slightly diminished with the other mutations. Mutation at these positions may affect to some extent the integrity of the C terminus of the receptor. In contrast, FF4L and the mutant receptors were equally precipitated by the GST-SH2 SHC recombinant protein (Fig. 5*B*, *left panel*). No receptor was precipitated either by GST protein from unstimulated cells or by GST alone from stimulated cells (data not shown). These results suggest that the Tyr^{1337} encompassing sequence constitutes a binding site for the PTB domain of SHC. The activated FF4S receptor bound to the recombinant GST-PTB protein only weakly (as for the .37F mutant, the low level of binding being probably in relation with a background binding of GST fusion proteins), although comparable amounts of receptor were detected in total lysates of each population (Fig. 5*A*). For an unknown reason, FF4S bound also only weakly to GST-SH2 SHC. It is possible that FF4L but not FF4S is appropriately phosphorylated at the SH2 binding site (20).

Interaction between the SHC PTB Domain and the Tyr(P)^{1337}-containing Sequence—A second approach was used to confirm the interaction between the SHC PTB domain and the Tyr(P)^{1337}-containing sequence. Four SHC cDNAs, coding respectively for the wild type p46 SHC and three mutant forms, were constructed. The four resulting proteins were tagged at their N terminus with an hemagglutinin (HA) epitope. They were tested for their binding to the peptides described above.

As the p46 and p52 SHC proteins diverge at their N terminus by 46 amino acids dispensable for the functional integrity of the PTB domain (22, 35, 36, 40), we only tagged the p46 SHC protein (Fig. 6*A*). The HA-dSH2 and HA-dPTB proteins contain deletions of the SH2 and PTB domain, respectively. In addition, Arg^{401}, a residue crucial for SHC SH2 binding to phosphotyrosine (47–49), was replaced by a leucine to produce the HA-L401 mutant. These mutants were cloned in the pECE expression vector for analysis in the Cos cells transient expression system. Expression of tagged recombinant SHC proteins was determined by immunoblot analysis with anti-HA monoclonal antibody. Proteins of predicted sizes were detected (Fig. 6*B*). As controls, Cos cells were transfected with a HA-pECE construct (HA), and with a HA-M2K pECE (HA-M2K) construct containing the full-length MAPKK cDNA (46). The membrane was probed with anti-SHC SH2 antibody (22), which, as expected, only detected HA-p46, HA-L401, and HA-dPTB proteins (data not shown). Tagged SHC proteins were then tested for their interaction with peptides containing the unphosphorylated or phosphorylated Tyr^{1337} residue (peptides 1 and 3, respectively) (Fig. 6*C*). HA-p46 was specifically precipitated only by the phosphorylated peptide. Mutation or deletion of the SHC SH2 domain (HA-L401 and HA-dSH2 mutants, respectively) did not significantly alter the efficiency of the interaction, whereas deletion of the PTB domain (HA-dPTB protein) completely abolished the association. An isolated PTB domain retained its capacity to associate with the Tyr(P)^{1337}-containing peptide (data not shown), which confirms that the Tyr(P)^{1337}-containing sequence represents a binding site for the SHC PTB domain. This interaction was specific, as no signal was detected with HA-M2K.

Abrogation of SHC PTB/FF4L Receptor Interaction Abolishes FF4L Transforming Activity—The amino acid sequence surrounding the Tyr^{1337} residue of FLT4L corresponds to a consensus PTB binding site. The specificity of the interaction is brought about by an hydrophobic residue (phenylalanine for the FLT4L receptor) at position −5 relative to the tyrosine, and by an asparagine residue at position −3 (32, 37–43). Published sequence alignments depicting the PTB domains binding site include a proline residue at −2; however, *in vitro* and *in vivo* analyses fail to attribute to it a role for PTB binding, in contrast to the −3 asparagine residue (40, 41). Thus, absence of a proline residue in the FLT4L motif does not prevent it from constituting a PTB binding site. Recent data additionally suggest that a PTB binding peptide preferentially adopts a β turn conformation in solution (42). The Tyr(P)^{1337}-containing se-

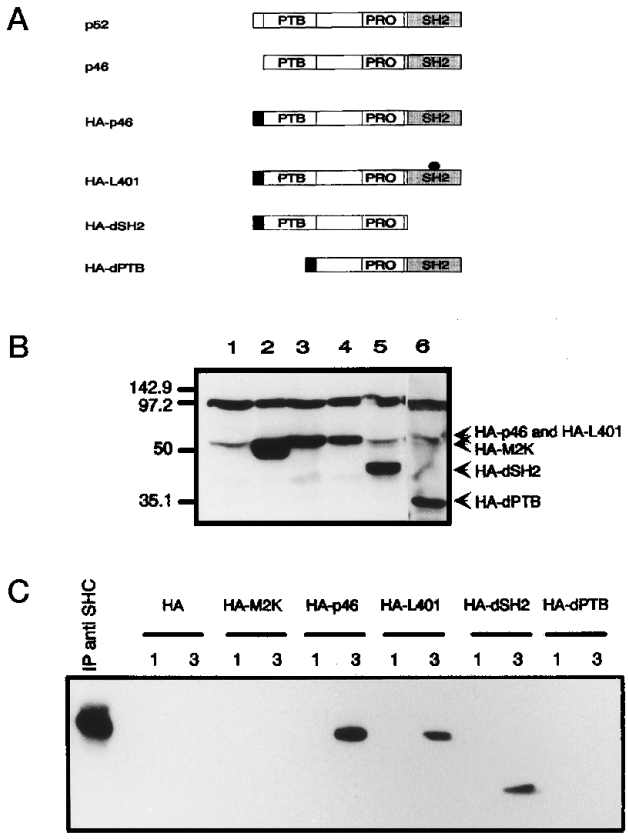

FIG. 6. **SHC interacts with FLT4L through its PTB domain.** *A*, the p52 and p46 SHC proteins are represented; the two tyrosine phosphorylated binding domains (PTB and SH2) flank a collagen homology domain containing proline-rich sequences (*PRO*). The respective epitope-tagged proteins (the HA1 epitope is represented by an N-terminal *black box*) are named HA-p46 (full-length p46SHC protein), HA-L401 (full-length p46SHC protein substituted at position Arg^{401} by a Leu residue abolishing the SH2 domain function), HA-dSH2 (SH2 deleted p46SHC protein), and HA-dPTB (PTB deleted p46SHC protein). *B*, Cos1 cells were transiently transfected with equal amount of SHC cDNA constructs and quantified lysates were electrophoresed on a 10% SDS-PAGE. The membrane was probed with monoclonal anti-HA antibody. *Lane 1*, pECE-HA (negative control); *lane 2*, pECE-HA-M2K (positive control) (46); *lane 3*, pECE-HA-p46; *lane 4*, pECE-HA-p46L401; *lane 5*, pECE-HA-dSH2; *lane 6*, pECE-HA-dPTB. *C*, equal amounts of Cos1 cell lysates were incubated with coupled peptide 1 (*1*) or peptide 3 (*3*) beads. After extensive washing, bound proteins were electrophoresed on a 10% SDS-PAGE, blotted onto Immobilon, and revealed with anti-HA antibody. The *first lane* represents a positive control (HA-p46 SHC protein precipitated with anti-SHC antibody).

quence indeed possesses a calculated index compatible with such a structure (data not shown). Since we have shown that $Tyr(P)^{1337}$, which is also essential for the receptor transforming capacity, is a SHC PTB binding site, then substitution of the −3 asparagine (Asn^{1334}) should abolish the transforming phenotype like the Tyr^{1337} mutation (20). The Asn^{1334} residue was substituted to alanine residue in the FF4L receptor.

The mutant receptor was tested for its interaction with SHC and for its behavior in soft agar assay. When the corresponding receptors were expressed at similar levels in infected Rat2 cells (Fig. 7*A*), the Tyr^{1337} and N^{1334} mutations similarly decreased SHC phosphorylation and abolished the co-immunoprecipitation of the stimulated receptor (Fig. 7*B*), although identical amounts of SHC proteins had been loaded in the gel (Fig. 7*C*). This effect could not be attributed to a default in FF4L.34N receptor tyrosine kinase activity because it could be phosphorylated as efficiently as the wild type FF4L receptor (data not shown).

Different populations of Rat2 cells were seeded on soft agar in the presence or absence of CSF1. Only those expressing the wild type FF4L, and the FF4L.33F and FF4L.63F mutated receptors were able to promote the growth of colonies in the presence of ligand (Fig. 7*D* and data not shown). Cells expressing either no receptor (not shown), FF4L.34N (Fig. 7*D*), or FF4S receptor (data not shown) were unable to promote anchorage independent growth. In addition, we mutated the Leu^{1340} residue (residue +3 from the Tyr^{1337} residue), which could be involved in a potential SH2 SHC binding; we only observed a small decrease in the colony size in soft agar but no significant difference with wild type FF4L receptor (data not shown). Thus the Tyr^{1337} and N^{1334} mutations dramatically interfere with the association of the FF4L receptor with the SHC proteins, and therefore with its transforming activity. This suggests a central role of the association between FLT4L and the PTB domain of SHC in the transforming activity of the receptor.

DISCUSSION

Receptors with tyrosine kinase activity transduce a signal to the nucleus through a complex network of interactions with cytoplasmic proteins. Present on the latter, SH2 and PTB domains interact with phosphorylated tyrosine-containing sequences of the receptors (30, 50). Very few primary amino acid similarities exist between PTB and SH2 domains. It is speculated that novel structural features determine PTB/phosphotyrosine peptide interactions. This is supported by the three-dimensional model of the interaction of the PTB domain with its peptide ligand (51).

FLT1/VEGFR1 and FLK1/VEGFR2 are high affinity endothelial receptors for factors of the VEGF family (7, 13–16). They can produce different biological effects mainly attributed to their divergent transducing signal pathways (11, 12, 17, 52). FLT4/VEGFR3 possesses about 30% identity in its extracellular region with VEGFR1 and VEGFR2 (4, 21). It is expressed as two isoforms (FLT4S and FLT4L), differing by 65 C-terminal amino acids present only in the long form (19, 20). The long form is endowed with a transforming activity, and this is attributed to its capacity to phosphorylate the SHC protein.

In this paper, we show that the binding to the PTB domain of SHC is essential for the transforming capacity of the FLT4 receptor. We have investigated the interaction between FLT4L and SHC proteins and the potential role of the SHC PTB domain in this interaction. We observed co-immunoprecipitation of SHC isoforms and FLT4L during the first 10 min of FLT4L activation. In contrast, FLT4S is unable to associate with SHC proteins. Mutational analysis of FLT4L previously showed that the $Tyr^{1337} \rightarrow$ Phe mutation abolishes both FLT4L transforming activity and SHC phosphorylation (20). Using three different approaches, we show here that the Tyr^{1337} residue constitutes a binding site for the PTB domain of SHC *in vivo* and *in vitro*. (i) The Tyr^{1337} to F^{1337} mutated FLT4L receptor lose its capacity to associate with SHC. (ii) A $Tyr(P)^{1337}$-containing peptide (but not a $Tyr(P)^{1333}$ peptide) was able to specifically bind SHC proteins *in vitro*. (iii) GST-PTB SHC recombinant and epitope-tagged SHC proteins were used to confirm this interaction; Tyr^{1337} mutation (but not Tyr^{1333} or Tyr^{1363} mutations) decreased FLT4L capacity to interact with PTB-SHC recombinant proteins, and the deletion of SHC PTB domain (but not of SHC SH2 domain) abolished the binding of SHC to $Tyr(P)^{1337}$-containing peptide. Thus, the Tyr^{1337} residue constitutes an *in vivo* and *in vitro* binding site for the PTB domain. The Tyr^{1337} residue lies in the F-*X*-N^{1334}-*X*-*X*-Y^{1337} sequence matching a consensus PTB binding site (32, 39, 41–43). We therefore speculated that the $Asn^{1334} \rightarrow$ Ala mutation could inactivate the FLT4L transforming capacity as efficiently as the Tyr^{1337} mutation. Indeed, we found that

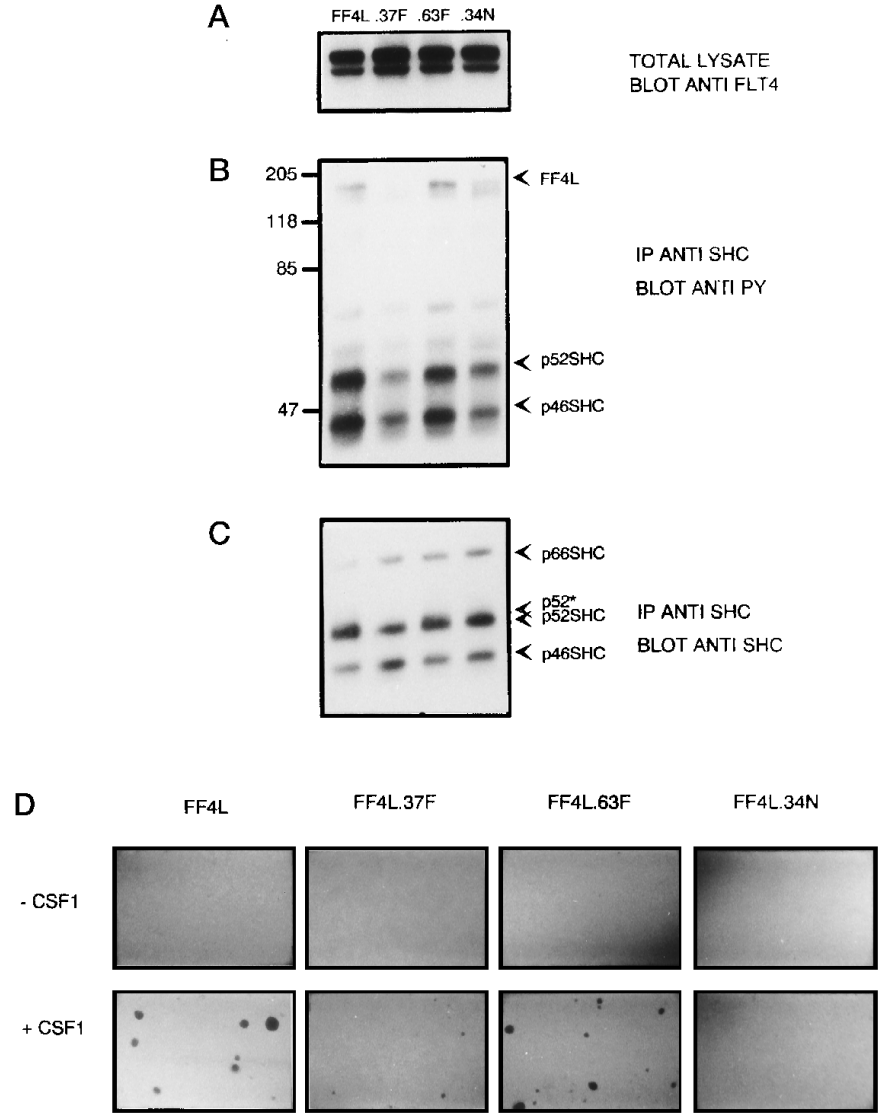

FIG. 7. **Interaction of SHC PTB domain is essential for the transforming activity of the receptor.** *A–C*, infected Rat2 cell populations (FF4L, FF4L.37F, FF4L.63F, and FF4L.34N) were starved overnight and stimulated with CSF1 (0.5 $\mu g \cdot ml^{-1}$) during 5 min. After lysis, an aliquot of lysates was electrophoresed and the membrane was revealed with anti-FLT4 antibody (*A*). The remaining lysates were immunoprecipitated with anti-SHC antibody. After migration and transfer, proteins were revealed with anti-phosphotyrosine (*B*) or anti-SHC (*C*) antibody. *D*, infected Rat2 cell populations expressing equal amount of chimeric molecules (as determined by fluorescence-activated cell sorting analysis; data not shown) were seeded on soft agar in the absence (*−CSF1*) or presence (*+CSF1*) of CSF1 growth factor. Colonies were photographed after 15–21 days of growth depending on experiments.

the $Asn^{1334} \rightarrow$ Ala mutant had a decreased capacity to phosphorylate SHC and could not induce the anchorage-independent growth of FLT4L-expressing Rat2 cells in a soft agar assay.

Although the Asn^{1334} mutation abolished FLT4L transforming activity *in vivo* (Fig. 7*D*), we observed a decrease but not a total inhibition of SHC proteins precipitation with a $Tyr(P)^{1337}$ peptide containing the same mutation (data not shown). These data support the idea that the tyrosine and the asparagine residues are not the only important residues involved in the PTB/peptide interaction (39–41) and that decreased affinity between both partners (SHC and FF4L) is sufficient to abolish FF4L transforming activity. Activation of FLT4L induced SHC tyrosine phosphorylation and retarded gel migration (p52*) (see Figs. 4*B* and 7*C*). The Tyr^{1337} but not the Asn^{1334} mutation abolished p52* formation, although both mutations abolished SHC tyrosine phosphorylation (Fig. 7*C*). The position 1337 mutation did not abolish a shift in mobility of the p66 SHC. It could thus be speculated that these shifted proteins result from serine/threonine phosphorylations. The tyrosine and serine phosphorylations allow SHC proteins to interact with different proteins connected to different transducing pathways (26, 53, 54). In the case of FLT4L, the tyrosine phosphorylation of SHC seems crucial for transforming activity. Tyr^{317} constitutes the major SHC phosphorylation site and the binding site for SH2 domain of GRB2 domain. This interaction promotes cellular transformation by providing a link to the RAS pathway through the recruitment of the SOS GDP/GTP exchange factor (33, 55–61). The decreased amount of SHC·GRB2 complex found in FF4L.37F could thus be in direct relation with its inability to transform cells.

The polyoma virus middle T antigen (MTAg) and the NEU/ERBB2 receptor possess a transforming activity which requires an intact N-P-*X*-(p)Y sequence able to bind the SHC PTB domain (23, 36, 42, 43, 62, 63). The TRKA receptor induces differentiation of PC12 cells through the binding of SHC PTB domain to Tyr^{490} residue and the subsequent SHC phosphorylation (40, 64). In contrast to NEU/ERBB2 and FLT4L, MTAg and TRKA proteins are unable to bind the SHC SH2 domain (20, 21, 65). FLT4L transforming activity is strictly dependent on Tyr^{1337} phosphorylation and on its association with the SHC PTB domain. Van der Geer and Pawson (30) proposed a model to describe the interaction between RTKs and SHC proteins through their SH2 and PTB domains. In line with this model, it is possible to speculate that the association of both the SH2 and PTB domains with FLT4L is crucial for the phosphorylation of SHC proteins and the generation of a transforming signal. Both domains interact with the FLT4L receptor, but it is not known whether they bind to the same molecule or promote interactions with two FLT4L receptors or with other cytoplasmic proteins. It is possible that the same SHC molecule

binds to FLT4L by both its domain. The binding of the PTB domain would influence that of the SH2 domain (this would explain the difference in intensity observed between FF4S and FF4L binding to GST-SH2 SHC). Specifically, the difference in the transforming capacity of FF4S and FF4L may thus be related to their differential capacity to activate the RAS pathway through SHC/GRB2/SOS.

FLT4 possesses the capacity to transduce different signals by using alternative processing of a primary transcript to promote the synthesis of a receptor containing or not a PTB binding site (FLT4L and FLT4S, respectively) (19). The same mechanism has been speculated for the MCK10b RTK but no direct data yet support this prediction (30). FLT1/VEGFR1 is also putatively expressed as two isoforms differing by an extension of 65 C-terminal amino acids (13). However, no tyrosine residue-containing sequence matching a PTB binding site is present in this additional stretch. FLT4 represents an example of RTK endowed with transforming activity strictly dependent on a SHC PTB domain binding and a model of RTK regulating its activity by alternative use of cytoplasmic substrates. Thus two types of possibilities are offered to cells to promote different cellular functions from few genes. First, some ligands (VEGF, platelet-derived growth factors, and fibroblast growth factors for example) have the capacity to bind several receptors with different biological functions (17, 66). Second, a single receptor gene can produce proteins with different biological functions by modulation of ligand (67–69) or substrate binding, through the use of additional domains in the intra- or extracellular regions (70, 71). FLT4 represents a good model for a receptor able to modulate its functions through alternative transcriptional processing leading to isoforms with different transducing properties.

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Interaction with the Phosphotyrosine Binding Domain/Phosphotyrosine Interacting Domain of SHC Is Required for the Transforming Activity of the FLT4/VEGFR3 Receptor Tyrosine Kinase

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