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Reduced phosphatase activity of SHP-2 in LEOPARD syndrome: Consequences for PI3K binding on Gab1

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Abstract LEOPARD (LS) and Noonan (NS) are overlapping syndromes associated with distinct mutations of SHP-2. Whereas NS mutations enhance SHP-2 catalytic activity, we show that the activity of three representative LS mutants is undetectable when assayed using a standard protein tyrosine phosphatase (PTP) substrate. A different assay using a specific SHP-2 substrate confirms their decreased PTP activity, but also reveals a significant activity of the T468M mutant. In transfected cells stimulated with epidermal growth factor, the least active LS mutants promote Gab1/PI3K binding, validating our in vitro data. LS mutants thus display a reduced PTP activity both in vitro and in transfected cells.

Keywords: LEOPARD syndrome; *PTPN11*; SHP-2 phosphatase activity; Gab1; PI3K

1. Introduction

LEOPARD syndrome (multiple *l*entigines, *e*lectrocardiographic conduction abnormalities, *o*cular hypertelorism, *p*ulmonary stenosis, *a*bnormal genitalia, *r*etardation of growth, sensorineural *d*eafness; LS; MIM#151100) is a rare genetic disorder characterized by multiple lentigines and other cutaneous abnormalities such as "café au lait" spots, dysmorphic facies, cardiac defects, growth retardation and increased risk of malignancy. Several clinical manifestations overlap those of Noonan syndrome (NS; MIM#163950), a common genetic disease also typified by facial dysmorphy, heart defects and growth retardation. However "café au lait" spots are rare and multiple lentigines are typically absent in NS.

Recently, germline missense mutations in the *PTPN11* gene were identified in both syndromes and are now causally linked to the diseases [1,2]. Somatic *PTPN11* mutations are also responsible for juvenile myelomonocytic leukemia [3]. *PTPN11* encodes SHP-2, a widely expressed protein tyrosine phosphatase (PTP) involved in intracellular signalling downstream of several growth factors, cytokines, and hormone receptors [4]. The structure of SHP-2 consists of two Src-homology 2 (SH2) domains (N-SH2 and C-SH2), a single PTP domain and a C-terminal hydrophilic tail. SHP-2 displays a low basal catalytic activity due to close interactions between the N-SH2 and PTP domains that keeps the phosphatase in an autoinhibited closed conformation. Its catalytic activation thus requires to open the molecule by releasing these interactions, which occurs when its SH2 domains bind to specific phosphotyrosine motifs on SHP-2 upstream signalling partners. These partners include the insulin receptor substrate 1 (IRS-1) and the Grb2-associated binder 1, Gab1. Both are adapter proteins rapidly phosphorylated in response to the stimulation of insulin or epidermal growth factor (EGF) receptors, respectively. Downstream of Gab1 or IRS-1, SHP-2 promotes the activation of the Ras/mitogen-activated pathway, through mechanisms that are still under investigation [4,5]. Besides this signal-enhancing function, SHP-2 can play a modulatory role in the activation of phosphoinositide 3-kinase (PI3K), at least downstream of the EGF receptor. Indeed, this receptor recruits PI3K by phosphorylating Gab1 on binding sites for p85, the regulatory subunit of PI3K, and it was recently shown that these sites are dephosphorylated by SHP-2 during EGF stimulation [6].

In NS, most of SHP-2 mutations described so far affect residues located in or close to the interaction surface between the N-SH2 and the PTP domains [7]. This is thought to disrupt the autoinhibited closed conformation of SHP-2, resulting in an increased basal PTP activity of most NS-associated SHP-2 mutants observed in vitro [8,9]. Thus the basis of NS pathogenesis is certainly due to activating, "gain-of-function" mutations of SHP-2, even though the SHP-2-dependent mechanisms altered by its NS-mutant derivatives remain to be identified. In LS, only nine mutations were reported to date, affecting seven different amino acids [7]. These residues are all located in the PTP domain, and, even if no functional data were reported yet, these mutations are predicted to be also "gain-of-function", due to the clinical overlap between NS and LS.

This work aimed to define the effect of LS mutations on SHP-2 phosphatase activity. Using molecular modelling, all LS mutations were strikingly located in the catalytic cleft of SHP-2. We

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Abbreviations: EGF, epidermal growth factor; IRS-1, insulin receptor substrate 1; LS, LEOPARD syndrome; NS, Noonan syndrome; PTP, protein tyrosine phosphatase; WT, wild type

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then explored, both in vitro and in intact cells, the effect of three representative LS mutations on SHP-2 catalytic activity.

2. Materials and methods

2.1. Structural analysis of LS-associated SHP-2 mutant residues

The structure of wild-type (WT) SHP-2 [10] (PDB code 2shp) was loaded into the program ICM (Molsoft LLC, www.molsoft.com) and the LS-associated residues were mapped and visually inspected.

2.2. Phosphopeptide synthesis

The substrate phosphopeptide Gab1-pY589 (DSEEN-pY-VPMNPNL) and the activating peptide IRS1-pY1172 (SLN-pY-IDLDLVK) [8] were synthesized using small-scale Fmoc chemistry.

2.3. PTPN11 constructs

The human WT *PTPN11* cDNA and the mutants D61Y and C459G subcloned into the pcDNA6/V5-HisA vector were kindly provided by Bruce D. Gelb. The NS-associated D61del mutation [11] and the LS-associated mutations resulting in Y279C, T468M, and Q510P were introduced using site-directed mutagenesis (QuickChange, Stratagene) and verified by sequencing.

2.4. Cell lines, transfections and stimulation

Vero cells were grown, transiently transfected and stimulated with 30 ng/ml EGF as described [5].

2.5. Immune complex PTP assays

Transfected cells were harvested in lysis buffer: 25 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100 and Protease Inhibitor Cocktail (Sigma). SHP-2 was immunoprecipitated with anti-V5 antibody (Invitrogen) then washed in lysis buffer and in PTP buffer: 20 mM HEPES, pH 7.4, EDTA 1 mM, 5% glycerol, and 1 mM DTT. Immunocomplexes were resuspended in 50 μl of PTP buffer supplemented with 250 μM of Src-pY529 phosphopeptide (TSTEPQ-pY-QPGENL, Upstate Biotech) or 100 μM of Gab1-pY589 phosphopeptide alone or with 10 μM of the activating peptide IRS1-pY1172, and incubated for 30 min at 37 °C. To assess free phosphate release, supernatants were then placed in 96-well plates with Malachite Green solution (Upstate Biotech) and incubated for 15 min. Absorbances (630 nm) were compared to a phosphate standard curve [3,12].

2.6. GST-p85 affinity precipitation assay

This was performed as described [5].

3. Results and discussion

3.1. LS-associated SHP-2 mutated residues are clustered in the PTP catalytic cleft

The nine missense mutations reported to date in LS affect seven different amino acids localized exclusively in the SHP-2 PTP domain (Fig. 1A). To gain insight on LS mutations effects on SHP-2 function, we performed a structural model analysis of the residues which are found mutated in LS with respect to their positioning in the SHP-2 molecule [10]. Fig. 1B show that the seven residues are highly clustered in the catalytic core of the PTP domain, suggesting that LS mutations might alter SHP-2 catalytic properties, rather than modify its activation state as in the case of NS-associated mutations.

3.2. PTP activity of LS mutants is abolished when assayed using a standard PTP substrate

To study the effect of LS mutations on SHP-2 catalytic activity, we introduced by site-directed mutagenesis the mutations giving rise to the two common Y279C and T468M and the rare Q510P single amino acid changes in a plasmid encoding human WT-SHP-2. After transfection of these constructs in mammalian cells, we measured in vitro the catalytic activity of each mutant isolated by immunoprecipitation. Vero cells were chosen for their low basal level of SHP-2-dependent signalling pathways once incubated overnight in serum-free medium [5,13]. Following immunoprecipitation, the PTP activity of the different constructs was assayed using as substrate a synthetic phosphopeptide encompassing Y529 of Src, a standard in vitro substrate of PTP [3,9]. To test these mutants under stimulation by an upstream SHP-2 activator, the immunocomplexes were also incubated with 10 μM of a phosphopeptide containing the SHP-2-binding site of IRS-1 (IRS1-pY1172) [8]. As shown in Fig. 2, addition of IRS1-pY1172 phosphopeptide resulted in a 2–3-fold increase of WT-SHP-2 catalytic activity, attesting that these experimental conditions allow to detect SHP-2 activation. As additional controls, we verified that the well-characterized catalytically dead C459G construct displayed no PTP activity, and that the leukemia-associated D61Y mutant and of the NS-associated D61del mutant have an increased PTP activity compared to WT-SHP-2. These two mutants are not significantly overstimulated by the IRS1-pY1172 peptide in these conditions, but this is in agreement with a previous report [8]. In addition, when the immunocomplexes were incubated with the activating peptide alone, the release of phosphate was undetectable for any of SHP-2 construct (data not shown), indicating that this phosphopeptide was not significantly dephosphorylated by SHP-2 when used in these conditions appropriate to observe its activating effect. This series of controls validated the methodology used in this study.

More interestingly, when considering the three LS-associated SHP-2 mutants, Fig. 2 shows that their basal catalytic activity is similar to that of the catalytically dead C459G construct. In the presence of the activating peptide, a slight but significant increase of PTP activity was detected in the case of Y279C and T468M mutants, but this activity remains at the most around 10% of that of WT-SHP-2. Taken together, these results suggest that LS mutations lead to the loss of SHP-2 catalytic activity, probably by disruption of the integrity of SHP-2 catalytic cleft.

3.3. PTP assay of LS mutants using a specific SHP-2 substrate confirms their decreased activity but reveals a significant activity of T468M mutant

The above results were obtained with a standard PTP substrate, which is not considered as an undeniable SHP-2 substrate in vivo. We thus determined if LS mutations had the same effect on SHP-2 catalytic activity when assayed on a validated SHP-2 substrate. To this aim, we designed a phosphopeptide encompassing Y589 of Gab1 (Gab1-pY589 phosphopeptide), which corresponds to a PI3K-binding site and is known to be dephosphorylated by SHP-2 in cells treated with EGF [6]. As shown in Fig. 3, the basal PTP activity of the three LS mutants assayed with this peptide is still severely reduced in comparison with the WT protein. However, in the case of the T468M mutant, its basal activity is around 30% of that of WT-SHP-2. Under stimulation with IRS1-pY1172, the Q510P mutant still displayed a negligible PTP activity. In contrast, the two other mutants seem to display a significant

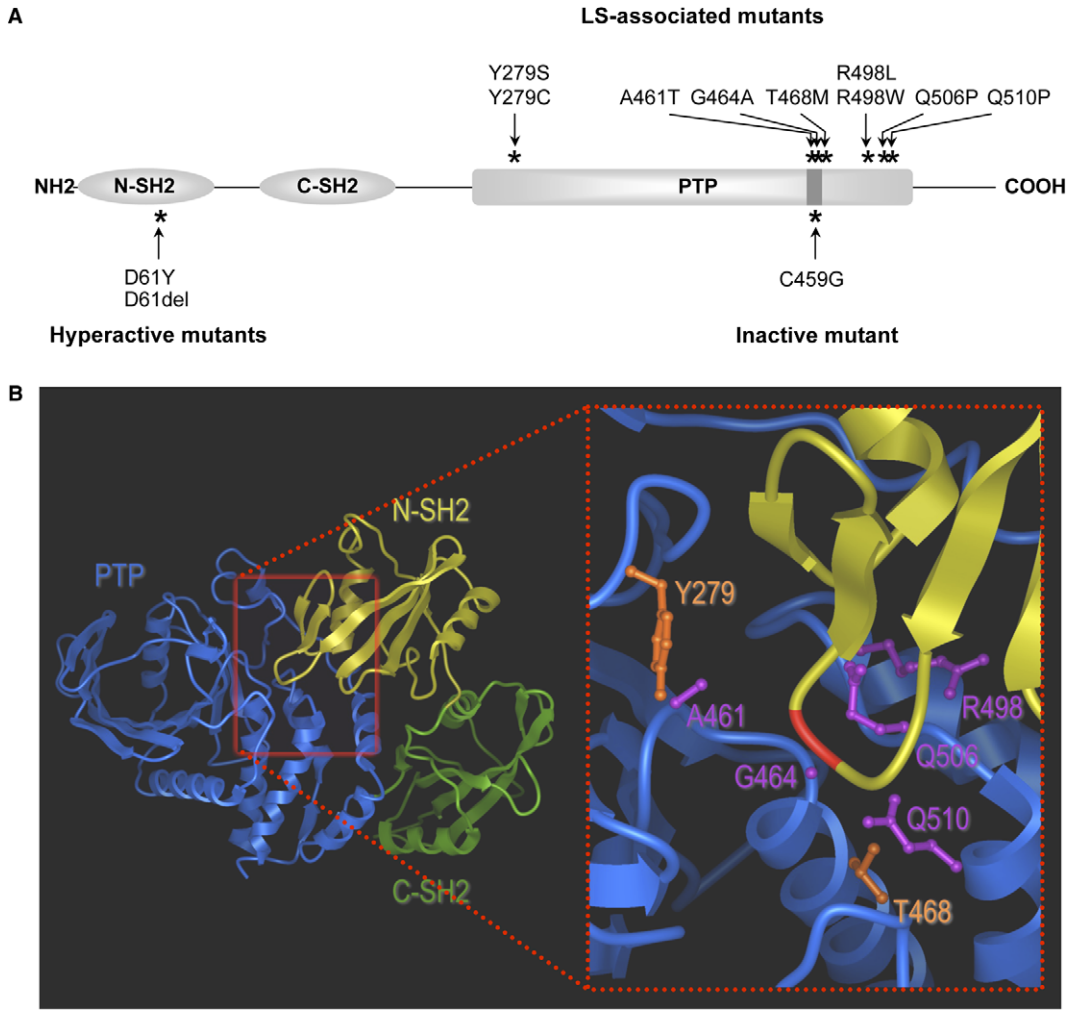

Fig. 1. LS-associated SHP-2 mutated residues are clustered in the PTP domain of the protein. (A) Schematic representation of SHP-2 showing the distribution of mutations identified in LS (above). Position of hyperactive and inactive SHP-2 mutants studied in this report are also indicated (below). (B) Left: ribbon representation of SHP-2 structure in its autoinhibited closed conformation [10]. The N- and C-terminal SH2 domains are coloured in yellow and green, respectively, the catalytic domain in blue. The red square depicts the closeup region. Right: Close-up of the active site and location of LS-associated SHP-2 mutated residues. The two most frequently altered residues Y279 and T468 are represented as orange sticks, while other mutations are shown as magenta sticks. Position of D61 (on the inhibitory loop of N-SH2 domain protruding into the catalytic site) responsible for autoinhibition of PTP activity is highlighted in red.

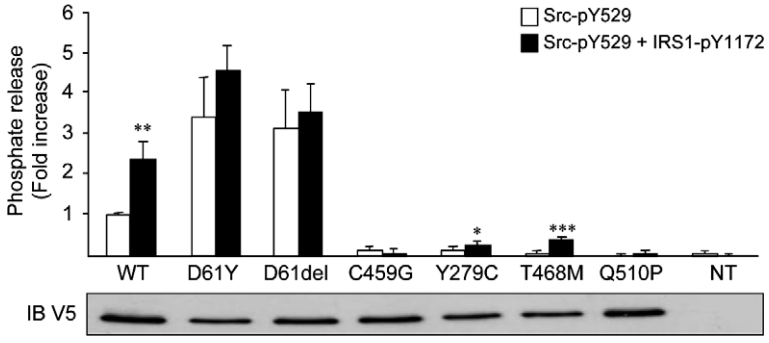

Fig. 2. PTP activity of LS mutants is abolished when assayed using a standard PTP substrate. Immune complex PTP assay against 250 μM Src-pY529 with or without 10 μM activating peptide IRS1-pY1172 carried out on serum-starved Vero cells transfected with the indicated SHP-2 construct. Bottom: representative anti-V5 immunoblots of immunoprecipitates. NT, experiments performed from non-transfected cells. Data represent means ± S.D. from three independent experiments. Significant differences in PTP activity between basal and activated conditions are indicated ($^{*}P < 0.05$, $^{***}P < 0.001$).

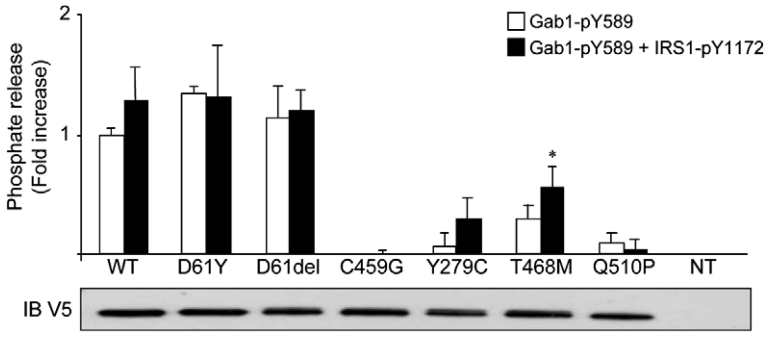

Fig. 3. PTP assays of LS mutants using a specific SHP-2 substrate. Immune complex PTP assays against 100 μM Gab1-pY589 with or without activating peptide IRS1-pY1172 carried out on serum-starved Vero cell transfected with the indicated SHP-2 construct. Bottom: representative anti-V5 immunoblot of immunoprecipitates. NT, experiments performed from non-transfected cells. Data represent means ± S.D. from three independent experiments. *, different from condition without IRS1-pY1172, $P < 0.05$.

PTP activity, notably the T468M that reaches 50% of WT SHP-2. These results thus confirm the Fig. 2 data showing that LS mutations, in contrast with NS mutations, lead to a decrease in SHP-2 catalytic activity. However, these experiments also indicate that different LS mutations produce distinct levels of alteration of SHP-2 activity, which was not detected using a standard PTP substrate. This suggest that at least one LS mutation influences SHP-2 substrate recognition rather than suppresses its catalytic activity.

3.4. *In situ confirmation of in vitro assays: the least active LS mutants promote Gab1/PI3K binding in EGF-stimulated cells*

We next determined whether these in vitro features of LS mutants could be confirmed in transfected cells, by testing the effects of these mutants on the dephosphorylation of a cellular substrate of SHP-2. Because SHP-2 can dephosphorylate Gab1 on its PI3K-binding sites in response to EGF [6], we designed an experiment to monitor these sites phosphorylation. Vero cells were chosen again, since they can be efficiently stimulated by EGF, leading to the phosphorylation of Gab1 and the downstream activation of SHP-2 and PI3K [5,13]. Following stimulation with EGF, cells were incubated with a GST-p85 fusion protein, which allows to precipitate Gab1 only if phosphorylated on PI3K binding sites [5,13]. As positive control, in cells transfected with catalytically dead C459G SHP-2, GST-p85 precipitates an higher amount of Gab1 in comparison with WT SHP-2, whereas, as negative control, the hyperactive D61del mutant prevents Gab1 precipitation (Fig. 4A–C, compare lanes "C459G" and "D61del" with "WT"). Thus Gab1 amount precipitated with GST-p85 appears inversely proportional to SHP-2 catalytic activity, showing that the Gab1/PI3K interaction can be taken as marker of the cellular catalytic activity of SHP-2.

Once validated, this assay was applied to cells expressing LS mutants. The results show that the Y279C and Q510P mutants enhance, compare to the WT-SHP-2, Gab1 recovery in GST-p85 pulldowns, measured either after 5 or 10 min of EGF stimulation (Fig. 4A and B). A quantitative analysis of data from different experiments indicates that these mutants produce an effect similar to that of catalytically dead SHP-2 C459G (Fig. 4D). In contrast, the T468M mutant did not enhance Gab1 precipitation by GST-p85 in comparison with WT-SHP-2, implying that this mutant species retains some of its phosphatase activity towards Gab1 PI3K-binding sites (Fig. 4C and D). We conclude that the LS mutants Y279C and Q510P behave in situ like catalytically inactive mutants, which confirms our in vitro data showing that these mutants display the lowest PTP activity. In addition, the fact that the T468M mutant does not promote Gab1/PI3K interaction more than the WT protein also confirms the in vitro results since this mutation was found to reduce, but not to abolish, its catalytic activity on Gab1 phosphopeptide. It is thus likely that this mutation does alter the catalytic efficiency of the T468M mutant, but its residual activity appears sufficient to dephosphorylate Gab1 in EGF-stimulated cells.

The apparent lack of hyperactivity of D61 mutants in vitro on Gab1-pY589 substrate is probably due to the use of a concentration of Gab1-pY589 lower than that of Src-pY529 phosphopeptide, according to previous reports that utilized these substrates [3,12], which likely results in a more rapid substrate depletion of Gab1-pY589. In support of this view, the experiments in intact cells (Fig. 4) show that the D61del mutant displays hyperactivity toward Gab1 PI3K-binding sites, which include Y589.

During the preparation of this manuscript, it was reported that LS-associated SHP-2 mutants, including Y279C and T468M, have lost their catalytic activity when assayed on standard PTP substrates [14]. The data obtained from our experiments performed in identical conditions confirm these observations. However, we measured for the first time the PTP activity of LS mutants using a specific SHP-2 substrate. At least in the case of the T468M mutant, we were able to detect a significant PTP activity, an observation which was validated ex vivo by monitoring Gab1/PI3K interaction. This reveals the importance of substrate choice for in vitro measurements of SHP-2 catalytic activity.

In conclusion, in contrast to NS, LS-associated SHP-2 mutants display a reduced PTP activity. How these apparently opposite behaviours of SHP-2 mutants lead to clinically overlapped syndromes still remain to be explained. However, the observation that one mutation (T468M) might possibly alter the substrate specificity of SHP-2 suggests that an heterogeneity of biochemical features exists between the different mutants, which may well be the key to the understanding of the variable occurrence of the different symptoms associated to each of these syndromes.

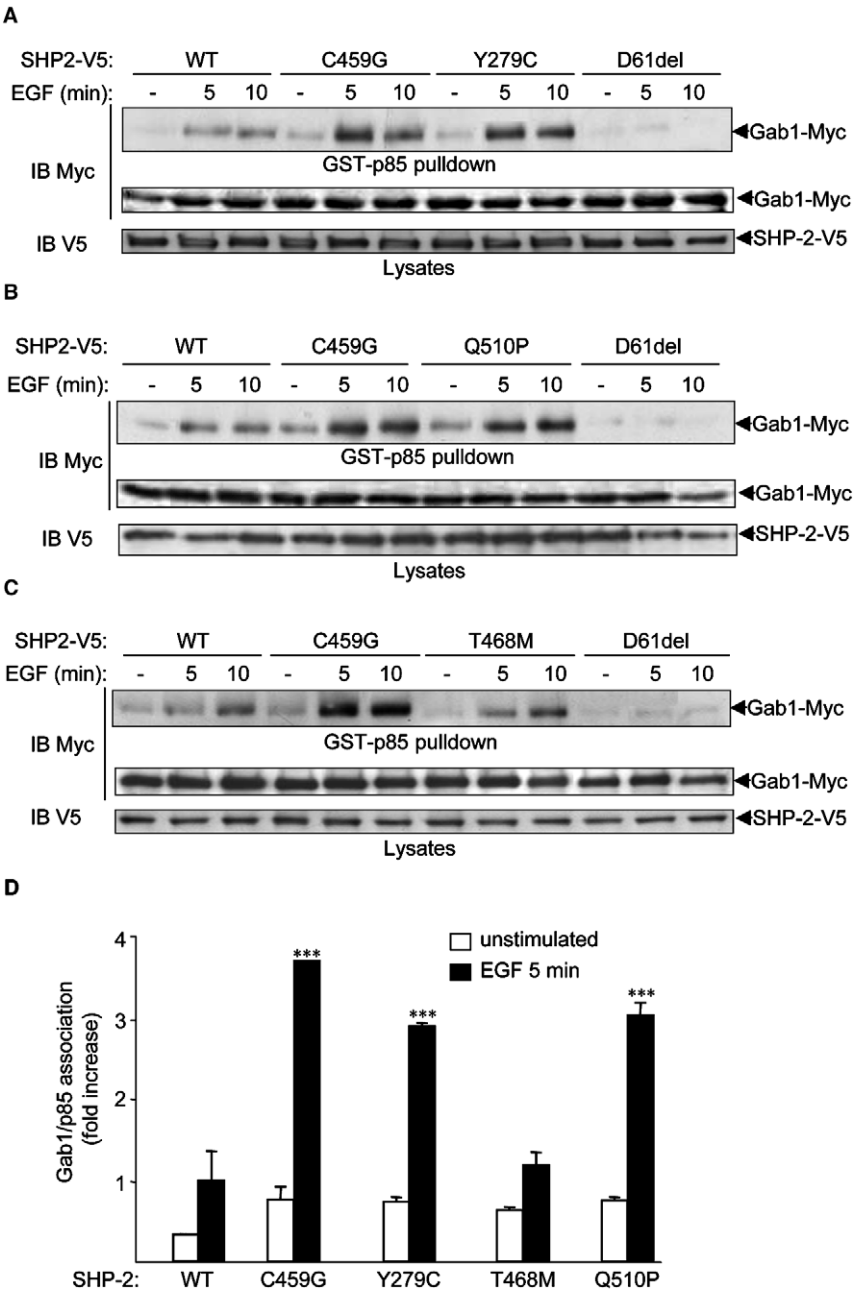

Fig. 4. Inactive LS mutants promote Gab1/PI3K binding in EGF-stimulated cells. (A–C) Vero cells cotransfected with Myc-tagged WT Gab1 and the indicated V5-tagged SHP-2 construct were stimulated with EGF when indicated. Cleared lysates were then subjected to a p85 affinity precipitation assay (''pulldown'') using a GST-p85α fusion protein. The amount of Gab1-Myc precipitated with GST-p85α has analysed by anti-Myc immunoblotting (top panels). Corresponding lysates were subjected to anti-Myc (middle panels) and anti-V5 (bottom panels) immunoblotting. (D) The immunoblots from three independent GST-p85 pulldown experiments were quantified using the software ImageQuant (Molecular Dynamics). Data represent means ± S.D. from three independent experiments. ***, different from WT (EGF 5 min), $P < 0.001$.

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