Published in final edited form as:
FEBS Lett. 2010 August 4; 584(15): 3279–3286. doi:10.1016/j.febslet.2010.06.029.

Abi1/Hssh3bp1 pY213 links Abl kinase signaling to p85 regulatory subunit of PI-3 kinase in regulation of macropinocytosis in LNCaP cells

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Abstract

Macropinocytosis is regulated by Abl kinase via an unknown mechanism. We previously demonstrated that Abl kinase activity is, itself, regulated by Abi1 subsequent to Abl kinase phosphorylation of Abi1 tyrosine 213 (pY213) [1]. Here we show that blocking phosphorylation of Y213 abrogated the ability of Abl to regulate macropinocytosis, implicating Abi1 pY213 as a key regulator of macropinocytosis. Results from screening the human SH2 domain library and mapping the interaction site between Abi1 and the p85 regulatory domain of PI-3 kinase, coupled with data from cells transfected with loss-of-function p85 mutants, support the hypothesis that macropinocytosis is regulated by interactions between Abi1 pY213 and the C-terminal SH2 domain of p85—thereby linking Abl kinase signaling to p85-dependent regulation of macropinocytosis.

Keywords

macropinocytosis; Abi1; spectrin; p85; Tyr213

Introduction

Macropinocytosis is an actin polymerization-dependent cellular process responsible for extracellular fluid and macromolecule uptake; however, the details of the mechanisms regulating macropinocytosis remain unclear [2,3]. In mammalian cells macropinocytosis is thought to be regulated by multiple pathways, which include several actin polymerization regulatory complexes, and involves receptor and non-receptor tyrosine kinases [4,5]. Abl kinases (Abl and Arg) are unique among cytoplasmic tyrosine kinases in their capacity to

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directly bind actin and to regulate actin polymerization [6,7]. Abl kinase activity is regulated by Abi1 [1,8]. The fact that overexpression of Abi1 inhibits macropinocytic uptake of fluorescent, water-soluble markers in NIH 3T3 [9] together with recent reports indicating that the anti-Abl drug Gleevec (STI-571) inhibits macropinocytic uptake of pathogenic bacteria [10,11], suggests the possibility that Abl tyrosine kinase activity is a key regulatory component of macropinocytosis. Moreover, expression of isoform 2 of Abi1, which inhibits Abl tyrosine activity [1], also reduces macropinocytic uptake in LNCaP cells [12].

Abl tyrosine kinase is implicated in regulating phosphotyrosine-mediated Abi1 interactions with several actin polymerization regulatory complexes [13] including PI-3 kinase [14]. PI-3 kinase involvement in regulating actin dynamics is well established [15], but is mostly attributed to the regulatory subunits of PI-3 kinase [16–18]. Studies have most often pointed to a critical role of the p85 subunit in formation of actin-rich membrane ruffles, which precedes fluid phase uptake into macropinocytic vesicles. PI-3 kinase activity was proposed to play a role in the closure of immature membrane ruffles to produce a macropinosome [19,20]. Wortmannin, an irreversible, PI-3 kinase inhibitor, inhibits macropinocytic uptake and the formation of membrane ruffles but not in all cells [21]. We previously demonstrated that wortmannin, and LY294002, a reversible PI-3 kinase inhibitor, inhibit macropinocytic uptake of Alexa Fluor 594 in NIH 3T3 cells [9]. Recent studies supporting the importance of PI-3 kinase and PIP signaling in the process [5] suggest that macropinosome maturation involves subsequent phosphatidylinositol 4,5-biphosphate -, and phosphatidylinositol 3,4,5-triphosphate -enriched stages [22,23].

Membrane ruffling [24] and enhanced macropinocytosis has been linked to enhanced Rac1 activation following PDGF stimulation [25]. In this regard, the growth factor-dependent activation of Rac1 occurs downstream of p85 interaction with the trimeric complex containing Abi1, Eps8, and Sos1 [14], which is mediated by the p85 N-terminal SH2 domain and Abi1 phosphotyrosine pY407 [14]. We hypothesized that the functional phosphotyrosine-dependent interaction of p85 with Abi1 involves Abi1 isoform 2, which binds active Rac1 [12]. Here we demonstrate that Abi1 isoform 2 is capable of multiple interactions with p85, but Abi1 pY213 and the p85 C-terminal SH2 domain specifically cooperate through a high affinity interaction to inhibit macropinocytic uptake in LNCaP cells.

Materials and Methods

Cell lines

LNCaP cells stably expressing Ha-tagged wild type Abi1 or Abi1 Y213F mutant [1] of isoform 2 (NM_001012750.1) [26] were grown in RPMI 1640 with 10 mM Hepes, 1 mM sodium pyruvate, and 2 mM glutamax (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).

Antibodies and reagents

Alexa Fluor 647 hydrazide, tris (triethylammonium) salt and Lipofectamine 2000 Transfection Reagent were from Invitrogen (Carlsbad, CA). PDGF BB (cat # 3201), wortmannin and latrunculin A were from Sigma-Aldrich (St. Louis, MO). Glutathione Sepharose 4B was from Amersham Biosciences (Piscataway, NJ). Imatinib mesylate (STI-571) was from Bioffin GmbH, Germany. Horseradish peroxidase-labeled goat anti-rabbit IgG, goat anti-mouse IgG and Protein G plus agarose were from Thermo Fisher Scientific (Worcester, MA). Monoclonal antibody to HA and His6 epitopes were from Roche Diagnostic Corporation (Indianapolis, IN). Polyclonal anti-GFP antibody was from Invitrogen. Rabbit antibody to p85 (clone 19H8) was from Cell Signaling Technology

(Danvers, MA). Protease inhibitor cocktail set I and set III were from EMD Chemicals Inc. (Gibbstown, NJ).

p85 expression plasmids

The loss-of-function p85 SH2 domain mutations, R358L (within the N-terminal SH2 domain), or R649L (within the C-terminal SH2 domain) [27], were introduced into the full-length p85-GFP (mouse), using Quick Change XL site directed mutagenesis kit (Stratagene, La Jolla, CA).

Recombinant Abi1 purification and affinity binding assay was performed as described [12]

Cells were transfected with wild type p85 or its mutants and subjected to immunoprecipitation and evaluation by Western blotting as described [9,26] except that NuPAGE gels (Invitrogen, Carlsbad, CA) were used.

Flow cytometric uptake assay of Alexa Fluor 647

Assay of cellular uptake of Alexa Fluor 647 was performed essentially as described [9], but was quantified by flow cytometry [12]. For uptake assays, following seeding into 12-well for 24 hours and washing, cells were preincubated with 0.2 μM wortmannin or 2 μM imatinib mesylate (STI-571) (30 min), and incubated in the same medium with or without 10 ng/ml PDGF for 2 hours along with Alexa Fluor 647 hydrazide (25 μM). In similar experiments in which cells were treated with 0.2 μM latrunculin A, PDGF was not added. Following washing with PBS (37°C) harvested cells (0.25% trypsin, 1 min) were subjected to cytofluorimetric analysis (BD FACS Canto, Becton Dickinson, San Jose, CA) in which the relative cellular content of Alexa Fluor 647 was measured by fluorescence intensity of the internalized dye in live cells. Intensity was obtained as a mean per cell value for 10^4 cells in each measurement. In transfection experiments with GFP tagged proteins, a positive Alexa Fluor 647 signal was evaluated only in GFP positive cells. For immunoprecipitations, cells were incubated with 100 ng/ml PDGF for 5 minutes.

Reverse-phase SH2 domain binding assay [28,29] is described in Supplementary Material

Immunofluorescence microscopy—For microscopic observations, cells were grown on glass bottom dishes (MatTek Corporation, Ashland, MA), treated as for flow cytometric uptake, and fixed with warm 4% paraformaldehyde for 20 minutes. Representative images were collected using a Zeiss Meta 510 Confocal Laser Scanning Microscope with a 100x NA oil objective. Images were processed using Adobe Photoshop CS3.

Data analysis—The significance of the data was analyzed using One Factor Anova. The Holm-Sidak test was used for comparison versus a control group. Differences with P value < 0.05 were considered significant (marked as a star in figures). Error bars represent the standard error of the mean (s.e.m.).

Results

High affinity interaction between Abi1 phosphopeptide pY213 and p85 regulatory subunit of PI-3 kinase

We previously demonstrated that tyrosine 213 (Y213) of Abi1 isoform 2 was an Abl kinase-dependent and Gleevec-sensitive phosphorylation site [1]. We hypothesized that after being phosphorylated at pY213, Abi1 would bind a downstream cellular ligand. We therefore searched for Abi1 pY213 interacting proteins by screening the genome-wide human GST-SH2 domain library as described [28]. As a comparison peptide we used an Abi1-derived peptide containing pY421 (Fig. 1A). Among proteins that bound with the highest affinity to

Abi1 pY213 was a recombinant polypeptide containing the N- and C-terminal SH2 domains of the p85 regulatory subunit of PI-3 kinase (Fig 1B) (Supplementary Figure S1). Unphosphorylated peptide controls demonstrated no appreciable affinity in the genome wide screening (Supplementary Figure S1). In addition, consistent with our previous observations, pY213 bound with relatively high affinity (but lower than to p85) to the Abl SH2 domain [1]. In contrast, pY421, showed high affinity binding to Fyn SH2 domain, weak binding to p85 and no binding to the Abl SH2 domain (Fig. 1). These data suggested that the Abi1 pY213 signaling pathway involved Abi1 interaction with p85 subsequent to Abi1 phosphorylation by Abl kinase.

Y213F mutation of Abi1 enhances macropinocytic uptake of Alexa Fluor 647

As indicated in *Introduction*, Abi1 and Abl kinase functions have been linked to regulation of macropinocytosis. The data that Abl kinase activity is regulated through Abi1 pY213 [1], provided rationale to determine the role of Y213 in macropinocytosis in the context of Abl kinase regulation. To do so we examined macropinocytic uptake of Alexa Fluor 647 in cells stably expressing the Abi1 Y213F substitution. Analysis indicated that cellular uptake of Alexa Fluor 647 was significantly enhanced in cells expressing Abi1 Y213F mutant in comparison to cells expressing wild type recombinant Abi1. Although Abi1 Y213F clone demonstrated PDGF response, the finding that Y213F mutation led to constitutive enhancement of Alexa Fluor 647 uptake (Fig. 2A and 2B) suggested that phosphorylation of Abi1 on pY213 negatively regulated macropinocytosis. Consistent with our recent findings, the fluorescent dye accumulated in large cell extensions [12], which are increased in numbers in cells expressing Abi1 Y213F (Supplementary Figure S2).

Y213F mutation changes sensitivity of Alexa Fluor 647 uptake to Abl kinase and PI-3 kinase inhibitors

Involvement of p85 regulatory subunit of PI3 kinase and Abl kinase activity prompted the use of specific inhibitors of the kinases to compare the uptake of Alexa Fluor 647 between LNCaP cell lines overexpressing Abi1 wild type or Y213F mutant. The abilities of wortmannin or of STI-571 treatment to inhibit macropinocytic uptake in LNCaP cells carrying the Abi1 Y213F mutation was abolished or reduced, respectively. Both LNCaP cell lines demonstrated a significant decrease in response to Latrunculin A, which is an actin depolymerization agent (Fig. 2A and B).

p85 C-SH2 domain co-operates with Abi1 pY213 to inhibit macropinocytic uptake of Alexa Fluor 647

Our earlier results indicated high affinity interaction of Abi1 pY213 with the p85 fragment containing both the N- and C- terminal SH2 domain. To determine if the up-regulation of macropinocytic uptake in cells expressing Abi1 Y213F mutant was mediated by loss of interaction between Abi1 pY213 and one of the p85 SH2 domains, as suggested by in vitro binding results with the mutant peptide Y213F, we analyzed Alexa 647 uptake in LNCaP cell lines transfected with the full-length p85 containing a loss-of-function mutation either in the N-terminal SH2 domain, p85 N-SH2 R356L, or in the C-terminal SH2 domain, p85 C-SH2 R649L (Fig. 2B). These mutations render p85 SH2 domains incapable of binding phosphotyrosine-containing ligands [27]. In control cells (mock transfected), expression of the C-SH2 domain mutant moderately enhanced macropinocytosis compared to cells expressing wt p85 or the N-SH2 mutant. In cells expressing Abi1 Y213F the C-SH2 mutant significantly enhanced uptake, but had no effect in cells expressing wt Abi1. Thus, the Y213F substitution resulted in dependence of uptake on the C-SH2 domain. Mutating the N-SH2 domain had only a moderate effect on reducing uptake in cells expressing wild type Abi1. In summary, these data indicate that Abi1 pY213 and p85 C-SH2 domain are

important in the context of full-length proteins to regulate macropinocytic uptake in LNCaP cells.

Abi1 pY213 interacts with p85 preferentially through the p85 C-terminal SH2 domain

To further confirm that the p85-Abi1 association is regulated by interaction between Abi1 pY213 and the C-SH2 domain, we analyzed protein complexes immunoprecipitated from LNCaP cell lines. The results indicated that the interaction between Abi1 Y213F and the full-length p85 was reduced by the C-terminal SH2 domain mutation to a greater extent than by the N-terminal SH2 domain mutation (Fig. 3A). These data also suggested that the interaction between Abi1 and p85 was primarily dependent on the presence of phosphorylated Abi1 Y213, because pY213 peptide exhibited a high affinity interaction with p85, but there was no binding of Y213F peptide to p85 in the screening (Supplementary Fig. S1). The interaction of the full length Abi1 containing Y213F mutation with p85 was also observed under the non-phosphorylating conditions, i.e., without PDGF (Fig. 3A). These data indicated that although pY213 is important for the interaction between Abi1 and p85, other interaction sites might be present in the complex.

To assess these candidate interactions we established affinities of Abi1 phosphopeptides to separate p85 SH2 domains in vitro. In addition to pY213 and pY421, we included pY506, which is another Abl kinase dependent site (Material and Methods). Affinity measurements indicated that while the isolated N-SH2 of p85 was capable of high affinity phosphotyrosine-dependent interaction with all three Abi1-derived peptides, the C-SH2 domain showed a preference for pY213 and pY506, with no measurable affinity for pY421 (Fig. 3B; see also Supplementary Fig. S3). Therefore, we postulated that the full-length Abi1-Y213F mutant might associate with p85 N-SH2 R356L through an interaction mediated by the intact C-SH2 and Abi1 pY506, and with p85 C-SH2R649L through an interaction mediated by the intact N-SH2 with pY506 or pY421. These candidate interactions might explain residual binding between Abi1 Y213F and the p85 mutant under phosphorylated conditions.

To understand the basis of Abi1-p85 interactions under non-phosphorylated conditions we analyzed the interaction of the recombinant polypeptides. We mapped the binding site of p85 to an Abi1 proline-rich and PXXP consensus containing fragment, residues 390–508 (Fig. 3C). These data raise the possibility that Abi1 Y213F interaction with p85 might be mediated by p85 SH3 domain (Fig. 3C). These data support the hypothesis that, although Abi1 interacts with p85 through multiple sites, the critical interaction regulating macropinocytosis uptake involves Abi1 pY213 and the p85 C-SH2 domain.

Discussion

In this report we have identified a critical link between Abl kinase and PI-3 kinase signaling that involves an adaptor function of Abi1. We demonstrate that Abi1 regulates macropinocytic uptake through an interaction with the p85 regulatory subunit of PI-3 kinase. The data indicate that Abi1 phosphotyrosine 213 co-operates with the C-terminal SH2 domain of p85 to inhibit macropinocytosis. We propose that Abi1 pY213 links Abl kinase signaling to p85-dependent regulation of macropinocytosis.

Abi1 Y213F mutation enhances macropinocytic uptake through abnormally regulated interactions with p85

Although p85 is capable of association with Abi1 Y213F, loss of the interaction with p85 C-SH2 domain seems to be critical for up-regulating macropinocytosis. Our in vitro binding data indicate that two candidate interaction sites for p85 N-SH2 domain remain on Abi1

Y213F i.e., pY421 and pY506. The latter is also capable of interacting with p85 C-SH2 domain. These data, together with the previously identified interaction of pY407 (numbering according to [14]) with the N-terminal SH2 domain of p85 [14], provides a rationale to explain the residual binding of the Y213F mutant under phosphorylating-conditions. The existence of multiple phospho-interaction sites on Abi1 suggest a complex mechanism of regulation, possibly involving competition among the phosphorylated tyrosines of Abi1 for binding to the p85 SH2 domains. We demonstrated that specific loss of Abi1 interaction with p85 C-SH2 domain, revealed here by Abi1 Y213F mutation, rather than the complete loss of the Abi1 interaction with p85, is critical for the regulation of macropinocytosis.

Based on the fact that Abi1 *wt* but not Abi1 Y213F, mediates a decrease in uptake in the presence of p85 C-SH2 domain mutation, we propose that Abi1 pY213 is a downstream mediator of a p85 C-SH2 domain signal. Our analysis here pertains to Abi1 isoform 2, which inhibits Abl kinase activity [1] and inhibits macropinocytosis, possibly through cytoplasmic sequestration of active Rac1 [12]. Direct interaction of active Rac1 with Abi1 is isoform-specific [12], raising the possibility of isoform-specific effects of p85-Abi1 interactions on Rac1 activation. Abi1 isoform-specific patterns of tyrosine phosphorylation, if different, might also contribute to the regulation of Abi1 interactions with p85. In this regard, more comprehensive analysis of tyrosine phosphorylation site mutants including tyrosine Y421 and Y506, in the context of Abi1 isoforms, will lead to better understanding p85-dependent regulation of macropinocytosis. In Abi1 isoform 2, tyrosine Y213 appears to play a critical role, as shown by the fact that introduction of Y213F mutation produces a similar effect on macropinocytosis as does siRNA-mediated downregulation of isoform 2, i.e., increased uptake [12].

Abi1 Y213 is phosphorylated by Abl kinase, thus suggesting modulation of macropinocytosis by Abi1-PI-3 kinase complex in Abl kinase-dependent manner. This hypothesis is consistent with proposed roles of tyrosine phosphorylation and phospho-inositol lipid signaling in actin cytoskeleton remodeling. Moreover, our novel data on p85-Abi1 interaction contribute to better understanding of regulatory mechanisms of macropinocytosis and suggest novel hypotheses regarding its regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Theresia Stradal for the p85-GFP plasmid, Elizabeth Luna for careful reading of the manuscript and discussions; and Ping Cui and Anna Chorzalska for technical help. This work was supported by grants R01NS044968 (NIH) and W81XWH-08-1-0320 (DOD) (L.K.). Xiaoling Xiong and Sajjad Hossain were supported in part by a grant (L.K.) from the F.M. Kirby Foundation, Inc., Morristown, NJ. Support from NIH grant CA107785 (to B.J.M.) and Breast Cancer Alliance (to K.M.) is also acknowledged.

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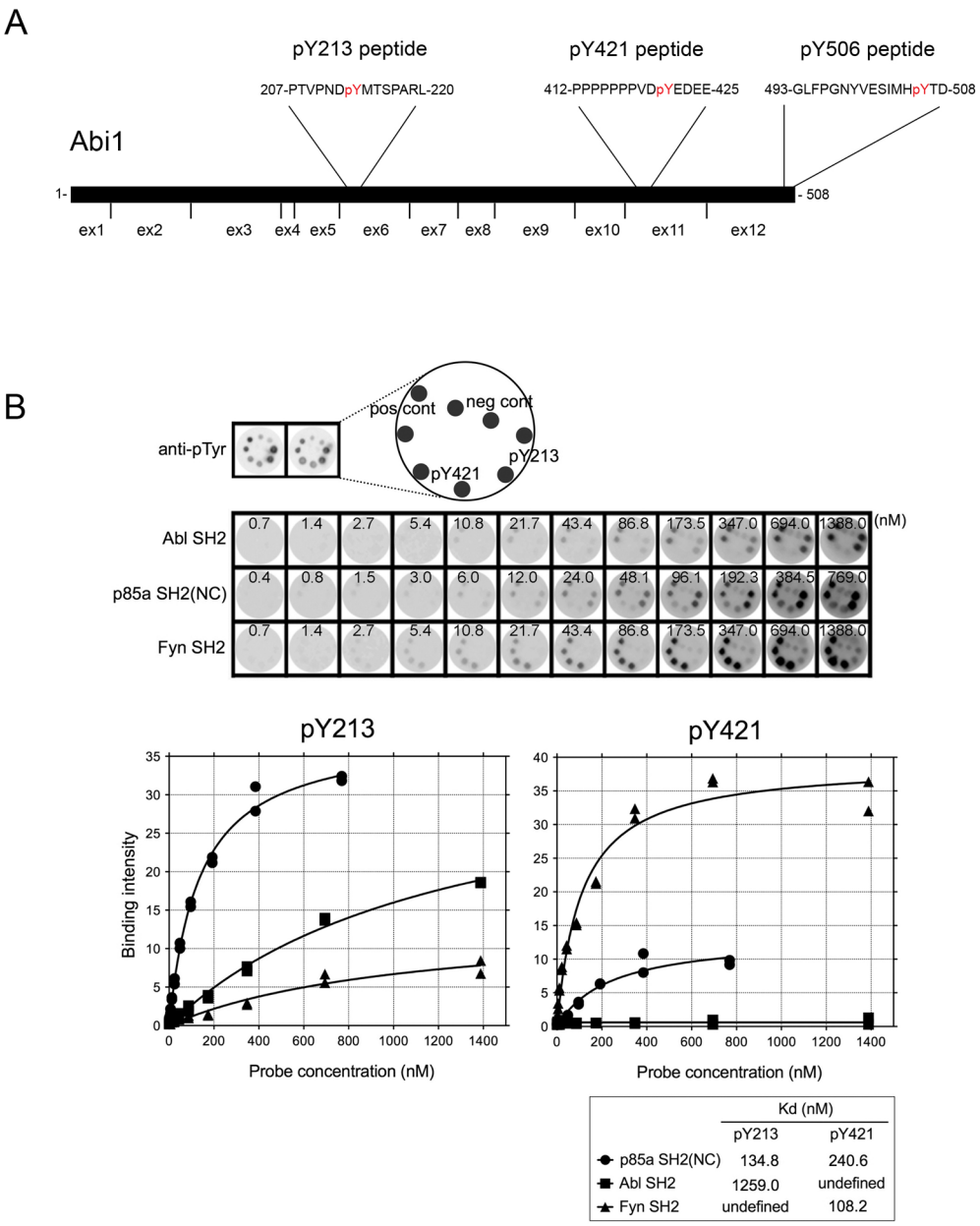


Figure 1. Identification of high affinity interactions of Abi1 with p85 regulatory subunit of PI-3 kinase through genome-wide SH2 domain phosphotyrosine peptide screening

A. Sequence and location of Abi1 phosphopeptides used in SH2 domain screening. Peptide sequences are listed above specific exons of the Abi1 schematic diagram; amino acid residue numbering is according to the full-length Abi1 (isoform 1, [26]). Isoform 2, lacking exon 8, was used in the study.

B. Identification of high affinity Abi1-pY213 interaction with p85. Synthesized phosphopeptides corresponding to pY213 and pY421 of Abi1 and controls were immobilized on a membrane in multiple duplicate arrays as shown on top. The assay positive control was a cocktail of lysates prepared from pervanadate-treated cells, and negative control a lysates with tyrosine phosphatase-treated [28]. An anti-phosphotyrosine blot is shown as a loading control. Labeled SH2 domains of Abl, p85 (containing both N-terminal and C-terminal SH2 domains), and Fyn were added into separate wells at indicated concentrations, and SH2

binding was detected by chemiluminescence. Quantified data were plotted against the SH2 probe concentration and equilibrium dissociation constants (Kd) values were determined by curve fitting. Affinities for some SH2-peptide interactions were not obtainable due to weak binding/low signal (undefined). For entire screening results including non-phosphotyrosine peptide controls see Supplementary Figure S1.

Figure 2A

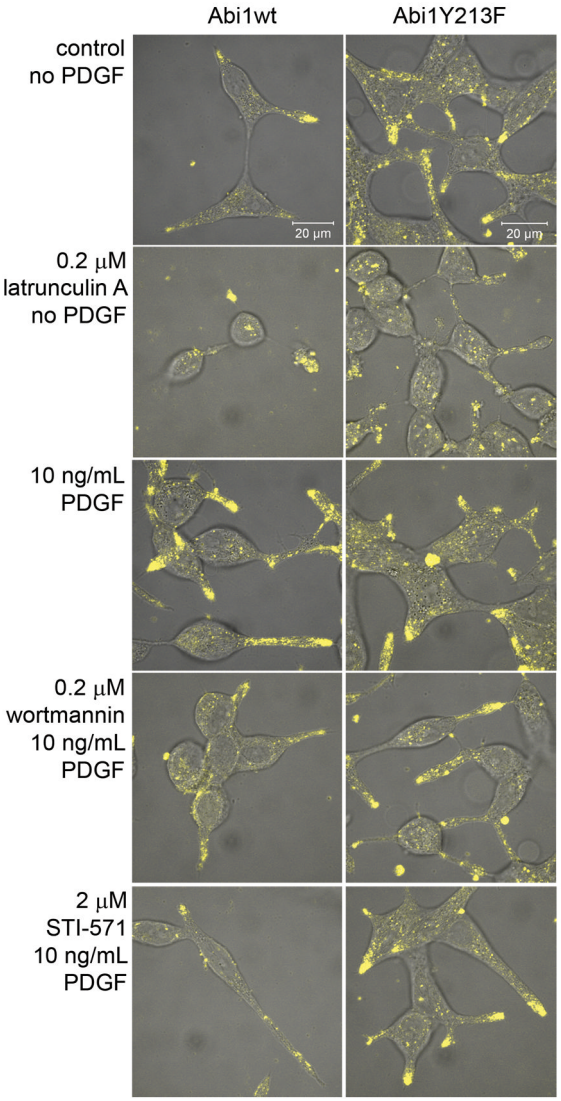
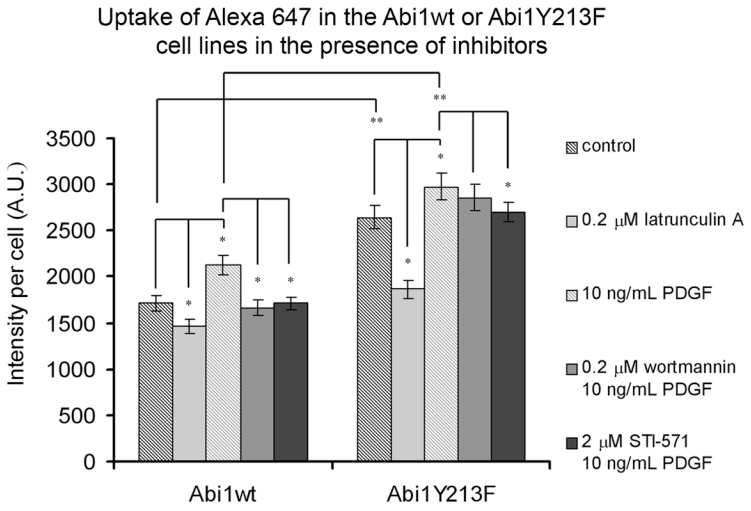


Figure 2BC

B



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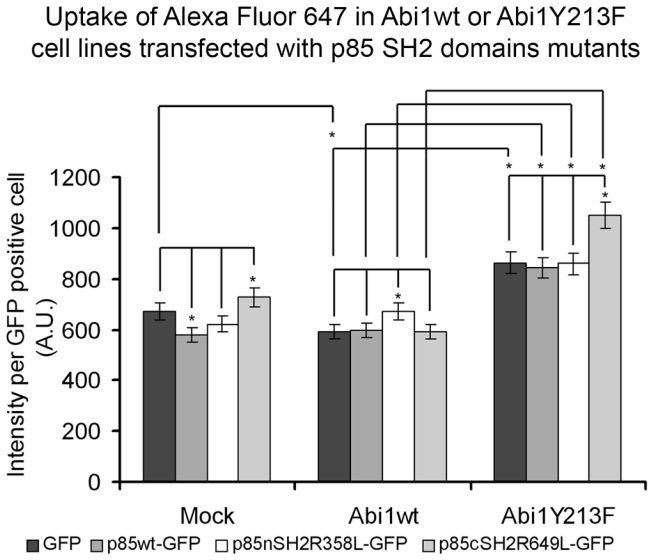


Figure 2. Abi1 regulates cellular uptake of Alexa Fluor 647 through tyrosine 213

A. Effect of actin polymerization, Abl and PI-3 kinase inhibitors on macropinocytic uptake. Representative confocal images from merged DIC and Alexa Fluor 647 (green) channels of LNCaP cells expressing wild type (Abi1wt) or Y213F mutation (Abi1Y213F). Cells were incubated with Alexa Fluor 647 for 2-hr following treatment with indicated agents as described in Materials and Methods.

B. Expression of Abi1 Y213F enhances cellular uptake of Alexa Fluor 647 and reduces sensitivity to inhibitors (wortmannin and STI-571). Measurements of Alexa Fluor 647 uptake were performed by flow cytometric assay in cell lines expressing wild type or Y213F mutant of Abi1 in the presence of PDGF and following treatment with indicated agents as described in Materials and Methods. Measurements were performed in triplicate, n=3, error bars ± s.e.m.

C. p85 C-SH2 domain mutation cooperates with Abi1 Y213F to enhance uptake of Alexa Fluor 647. LNCaP cells expressing wild type or Abi1 Y213F mutant of isoform 2 cells were co-transfected with p85 or p85-SH2 domain mutants as indicated. Abi1 isoform 2 inhibits macropinocytic uptake in comparison to Mock cells as previously observed [12]. Measurements were obtained in the presence of PDGF as described in Materials and Methods. Measurements were performed in triplicate, n=3, error bars ± s.e.m.

Figure 3A

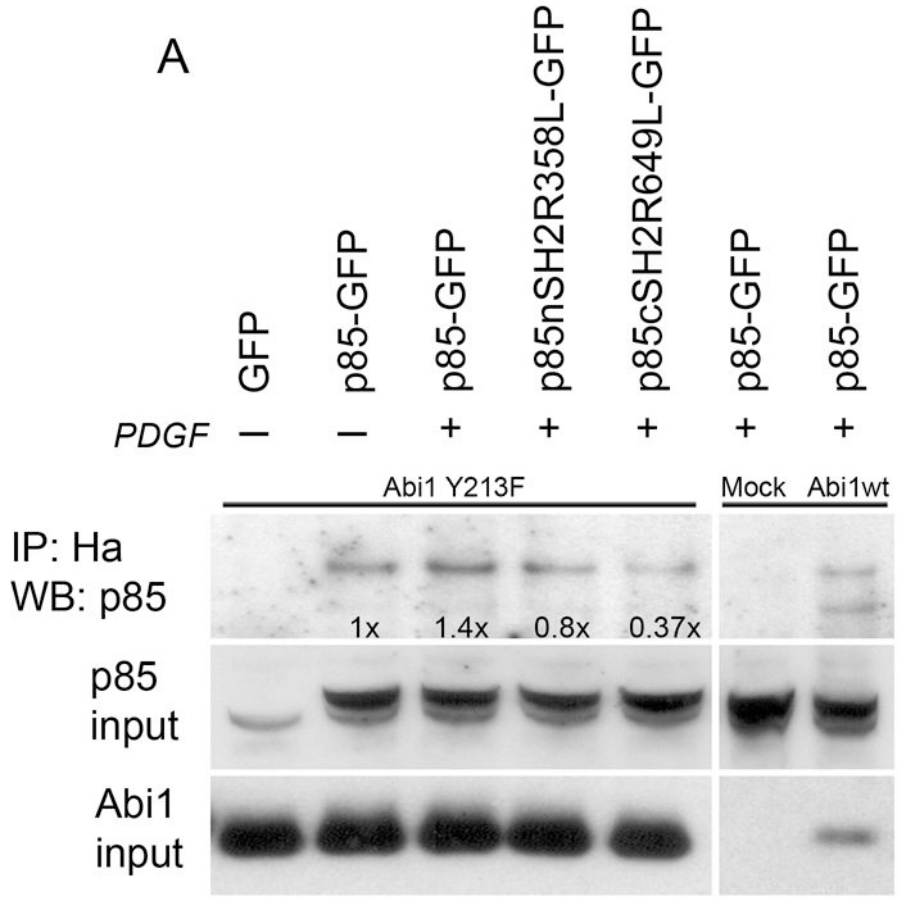
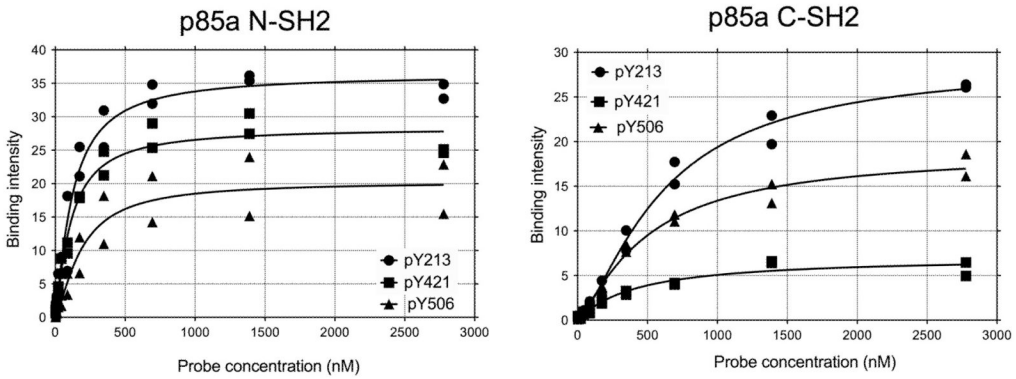


Figure 3B

B



Probe	Peptide	Kd (nM)
p85a N-SH2	pY213	120.9
p85a N-SH2	pY421	109.7
p85a N-SH2	pY506	184.7
p85a C-SH2	pY213	606.3
p85a C-SH2	pY421	undefined
p85a C-SH2	pY506	495.2

Figure 3C

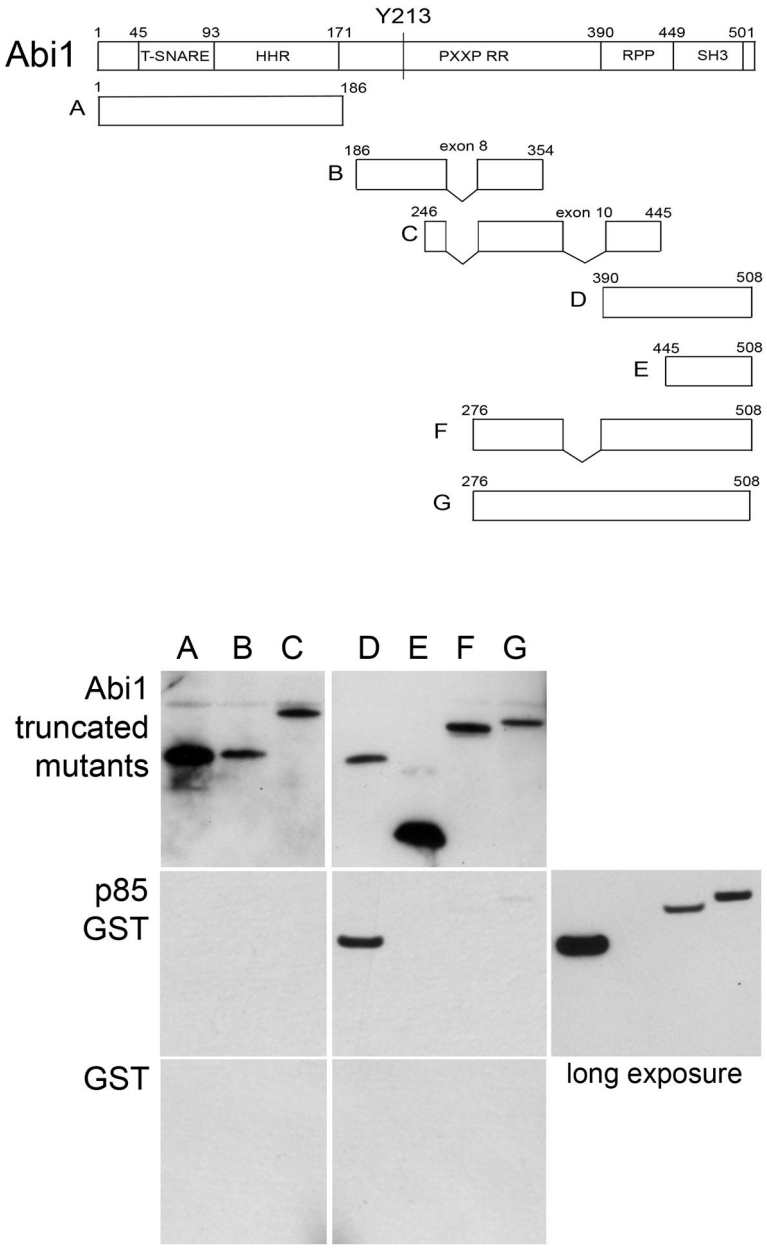


Figure 3. Abi1 pY213 interacts with p85 preferentially through the p85 C-terminal SH2 domain in transfected LNCaP cells

A. Interaction of Abi1 Y213F mutant with p85 is reduced by C-SH2 mutation. LNCaP cell lines expressing Abi1 *wt* or Y213F mutant were transfected with p85 or p85-SH2 domain mutants as indicated. Following immunoprecipitations using anti-HA antibody, samples were separated on SDS-PAGE gels and transferred as described in Materials and Methods. Membranes were blotted with anti-p85. Input indicates p85 levels in whole cell lysate; Abi1, levels of Abi1 in immunoprecipitated samples. Numbers within the top panel indicate relative band intensity normalized to the p85-GFP band with no PDGF. Immunoprecipitations were performed in the presence or absence of PDGF as indicated.

Fig. 3B. Affinities of Abi1 peptides to p85 SH2 domains. Affinity measurements were made using the assay [28] described in Fig. 1B and Material and Methods. Quantified data were plotted against the SH2 probe concentration and equilibrium dissociation constants (Kd) values were determined by curve fitting. Affinities of Abi1 phosphopeptides for the N-terminal (p85a N-SH2) and for the C-terminal (p85a C-SH2) domain of p85 are listed in table below graphs. Affinity of the pY421 to p85a C-SH2 domain interaction was not obtainable due to low signal (undefined). Sequence of Abi1 peptides used here are listed in Figure 1A.

C. Mapping of p85 interaction with recombinant Abi1. (top) Schematic representation of Abi1 truncated mutants used for mapping. A total of seven Abi1(His-tagged) constructs were used, each represents a major domain or a region in the Abi1 protein (as indicated). **(Bottom)** Bacterial lysates containing p85-GST and Abi1-His proteins were combined and lysed. p85-GST-interacting Abi1 polypeptides were evaluated following elution of the bound fraction from Glutathione Sepharose 4B column as described (Material and Methods). Western blotting results of the eluted samples: Abi1 recombinant protein input (anti-His antibody) (top panel); Abi1 polypeptides binding to p85-GST (anti-His antibody) (middle panel); and GST control (GST antibody)(lower panel). The mapping was done using recombinant peptides derived from Abi1 isoform 2 (lacking exon 8) and isoform 3 (lacking exon 10) as indicated in fragments. Abi1 contains several domains: T-SNARE, homeobox homology region (HHR), PXXP sequence-rich region (PXXP RR), Proline-rich and PXXP-rich region and (RPP), and an SH3 domain.