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A yeast two-hybrid study of human p97/Gab2 interactions with its SH2 domain-containing binding partners

Catherine Crouin[a], Mary Arnaud[a], Franck Gesbert[a,1], Jacques Camonis[b], Jacques Bertoglio[a,*]

[a] *Inserm Unit 461, Faculté de Pharmacie Paris-XI, 5 rue Jean-Baptiste Clément, 92296 Châtenay-Malabry, France*
[b] *Inserm Unit 528, Institut Curie, 26 rue d'Ulm, 75248 Paris, France*

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Abstract p97/Gab2 is a recently characterized member of a large family of scaffold proteins that play essential roles in signal transduction. Gab2 becomes tyrosine-phosphorylated in response to a variety of growth factors and forms multimolecular complexes with SH2 domain-containing signaling molecules such as the p85-regulatory subunit of the phosphoinositide-3-kinase (p85-PI3K), the tyrosine phosphatase SHP-2 and the adapter protein CrkL. To characterize the interactions between Gab2 and its SH2-containing binding partners, we designed a modified yeast two-hybrid system in which the Lyn tyrosine kinase is expressed in a regulated manner in yeast. Using this assay, we demonstrated that p97/Gab2 specifically interacts with the SH2 domains of PI3K, SHP-2 and CrkL. Interaction with p85-PI3K is mediated by tyrosine residues Y_{452}, Y_{476} and Y_{584} of Gab2, while interaction with SHP-2 depends exclusively on tyrosine Y_{614}. CrkL interaction is mediated by its SH2 domain recognizing Y_{266} and Y_{293}, despite the latter being in a non-consensus (YTFK) environment.

Key words: Src homology 2 domain; Phosphoinositide-3-kinase; SHP-2; CrkL; Gab2; Yeast two-hybrid

1. Introduction

p97/Gab2 is a recently characterized member of the large IRS/Gab family of scaffold proteins that become tyrosine-phosphorylated in response to a variety of growth factors, and integrate multiple signaling pathways. Gab2 was initially identified in hematopoietic cells, as a 97-kDa protein phosphorylated in response to various stimuli such as stimulation by cytokines, cross-linking of T- or B-lymphocyte receptors or transformation by Bcr/Abl [1–3]. We previously reported that p97/Gab2 is a major tyrosine kinase substrate in interleukin-2-stimulated human T-lymphocytes, and is associated in a stimulation-dependent manner with phosphoinositide-3-kinase (PI3K), with the tyrosine phosphatase SHP-2 and with the adapter protein CrkL [4]. Pull down experiments using a panel of GST fusion proteins allowed us to demonstrate that these interactions are mediated by the Src homology 2 domain (SH2) domains of these proteins recognizing tyrosine-phosphorylated Gab2. Subsequent to the cloning of murine and human Gab2 [5–7], sequence analysis revealed the presence of four putative recognition sequences (pYxxP, where pY stands for phosphotyrosine) for the CrkL SH2 domain, three putative recognition sequences (pYxxM) for the p85 PI3K subunit SH2 domains and two putative recognition sequences (pYxxV/L) for the SH2 domains of SHP-2. Since yeasts do not express endogenous tyrosine kinase, the conventional yeast two-hybrid system cannot be used to study SH2/phosphotyrosine interactions, unless one of the partners is itself a tyrosine kinase. Indeed, such an approach has been used to characterize the interactions of SHP-2 or IRS-1 with the insulin receptor kinase and with the IGF-I receptor, that autophosphorylate, and to study association of the Lyn tyrosine kinase with Cbl [8–10].

In an attempt to further characterize the interactions between Gab2 and its SH2-containing binding partners, we designed a modified yeast two-hybrid system in which the catalytic domain of Lyn, a member of the Src family of tyrosine kinases, is expressed in a regulated manner in yeast. Using this assay, we demonstrated that indeed p97/Gab2 interacts with the SH2 domains of p85-PI3K, SHP-2 and CrkL, but not with those of Shc or of either STAT5 isoforms. Interaction with p85-PI3K is mediated by tyrosine residues Y_{452}, Y_{476} and Y_{584} as predicted by Gab2 sequence analysis, while interaction with SHP-2 appears to depend exclusively on tyrosine Y_{614}. With regards to CrkL interaction, three of the four predicted sites appear not to be functional, and the CrkL SH2 domain only recognizes efficiently Y_{266} and Y_{293}, despite the latter being in an unusual (YTFK) sequence environment for CrkL–SH2 recognition.

2. Materials and methods

2.1. Plasmid constructs

cDNAs for the human CrkL SH2 domain (aa 1–127), tandem SH2s of human p85 α (aa 313–724) and of murine SHP-2 (aa 1–240) were excised from previously described pGEX vectors [4] and inserted in frame in appropriate derivatives of the pLexA vector. cDNA for human p97/Gab2 was amplified by polymerase chain reaction (PCR) from plasmid KIAA0571/AB011143 (a kind gift of Dr T. Nagase, Kazusa DNA Research Institute, Japan). Full length cDNA for human Gab2 was obtained by PCR of reverse transcribed Kit 225 cell

*Corresponding author. Fax: (33)-1-46 83 54 96.
E-mail: j.bertoglio@cep.u-psud.fr

[1] Present address: Institut Pasteur, Unité d'immunogénétique cellulaire, 25, rue du Dr. Roux, 75724 Paris Cedex 15, France.

Abbreviations: SH2, Src homology 2 domain; PI3K, phosphoinositide-3-kinase; IRS, insulin receptor substrate; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ONPG, 2-nitrophenyl-β-D-galactopyranoside

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RNA, using a 5′ primer oligonucleotide designed after the published sequence [7]. Full length cDNA as well as Gab2 subdomains were inserted in frame in a pGad vector whose multiple cloning site had been modified by oligonucleotide insertion. In some instances, as described below, the reverse constructs, i.e. Lex-Gab2 and Gad-p85 were used.

Mutations of tyrosine residues in Gab2 were performed using the Quikchange mutagenesis kit (Stratagene) according to the manufacturer's instructions.

For expression of the Lyn kinase in yeast, the cDNA encoding the tyrosine kinase domain of murine Lyn (aa 241–512) was amplified by PCR from a full length cDNA kindly provided by Dr. J. Cambier (Denver, CO, USA), tagged with the myc epitope and inserted in pMET416 under control of the methionine-regulated Met25 promoter [11].

All constructs, whether wild type or mutated, were verified by DNA sequencing.

2.2. Two-hybrid experiments

All yeast transformations were performed by the lithium acetate method. The TAT7 strain of *Saccharomyces cerevisiae (MATa, his3 leu2 trp1 ade2 LYS2::lex(op)-HIS3 ura3::lex(op)-LacZ)*, a gift from Dr R. Sternglanz (New York State University, NY, USA), was transformed with either pLex or pGad constructs or both, and transformants selected on appropriate selective medium (−Trp, −Leu). For introducing the Lyn kinase, TAT7 yeasts were mated with yeast strain W303-*1A/D (MATalpha his3 leu2 trp1 ade2 ura3)* transformed with pMET416-Lyn or empty pMET416 that contains a URA3 selection marker [12]. Following overnight mating on YPDA medium, patches were replicated on plates lacking Ura, Trp and Leu for selection of diploids. All interactions were tested in at least two independent transformations.

2.3. Filter and liquid β-galactosidase assays

For filter β-galactosidase assays, yeast patches were replica-lifted onto Whatman 40 filter paper and grown for 24 h. After freezing in liquid nitrogen, filters were laid over a Whatman 3 filter soaked in Z buffer (50 mM sodium phosphate buffer, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$) with 0.5% β-mercaptoethanol (βME) and 0.2 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Boehringer). After 2–6 h the reaction was stopped by transferring the filters to a 1 M Na_2CO_3 solution.

For liquid β-galactosidase assays, yeast was grown overnight on appropriate selective media and OD_{600} were recorded. Cells were pelleted by centrifugation from 1 ml aliquots of each culture and permeabilized by five cycles of freeze-thawing in 100 μl of Z buffer. Then, 700 μl of Z buffer plus βME and 150 μl of 2-nitrophenyl-β-D-galactopyranoside (ONPG, 4 mg/ml in H_2O) were added. The reaction was incubated at 30°C until color developed and OD_{420} was recorded. Results are expressed as Miller units: one unit of β-galactosidase was defined as $(OD_{420} \times 1000)/(OD_{600} \times \text{volume (ml)} \times \text{time (min)})$.

2.4. Western blot analysis

For Western blot analysis, yeast was grown overnight and recovered by centrifugation. Total yeast extracts were obtained by incubation in lysis buffer (Tris-buffered saline pH 8, 1% Triton X), followed by five cycles of freeze-thawing. Proteins (50 μg) were separated by SDS–PAGE and electro-transferred to nitrocellulose according to standard methods. The 9E10 antibody (anti-myc) and 4G10 (anti-phosphotyrosine) were used together with HRP-conjugated anti-mouse antibodies and ECL reagent as described. Cell extracts from starved or IL-2-stimulated Kit 225 cells were immunoprecipitated as previously described [4] with a Gab2 specific goat antiserum (sc-9313, Santa Cruz Biotech, CA, USA) and analyzed by Western blotting with indicated antibodies.

2.5. Peptide binding of CrkL

Biotinylated peptides designed after the sequence of human Gab2 were purchased from Sigma-Genosys. Peptides (5 μg), phosphorylated on tyrosine or not, were coupled overnight to streptavidin–agarose beads. Beads were washed and incubated for 2 h with Kit 225 cell lysates (800 μg) or GST–CrkL–SH2 fusion protein (5 μg). Beads were washed, boiled in sample buffer and the eluted material analyzed by Western blotting with anti-CrkL antiserum (sc-319, Santa Cruz Biotech., CA) or anti-GST antibodies.

3. Results

3.1. Tyrosine phosphorylation-dependent interaction of Gab2 with the SH2 domains of CrkL, PI3K and SHP-2

At the time of our previous report describing the phosphorylation of p97 in response to IL-2 stimulation in human T-lymphocytes [4], Gab2 had not yet been identified, and no specific antibodies were therefore available. We first wanted to confirm our previous findings using a Gab2 specific antiserum, and the data shown in Fig. 1 indeed clearly evidenced the tyrosine phosphorylation of Gab2 in IL-2-stimulated Kit 225 cells, and its interactions with p85-PI3K, SHP-2 and CrkL. To further analyze the interactions of p97/Gab2 with the SH2 domains of these proteins, we next established a modified two-hybrid system in which expression of the Src family kinase Lyn is repressed by the presence of methionine in the growth medium [11]. This was achieved by cloning the catalytic domain of Lyn under the control of the Met25 promoter on a centromeric URA plasmid. As seen in Fig. 2, when yeasts were grown in the absence of methionine, the kinase was expressed, as detected with the anti-myc antibody, and as a result a large number of yeast proteins became highly phosphorylated on tyrosine residues. It is of interest that expression of the kinase showed no deleterious effect on yeast growth. In the presence of 200 mg/l methionine, very little kinase was detectable (lanes 4 and 6, top panel) and tyrosine phosphorylation of endogenous substrates was minimal.

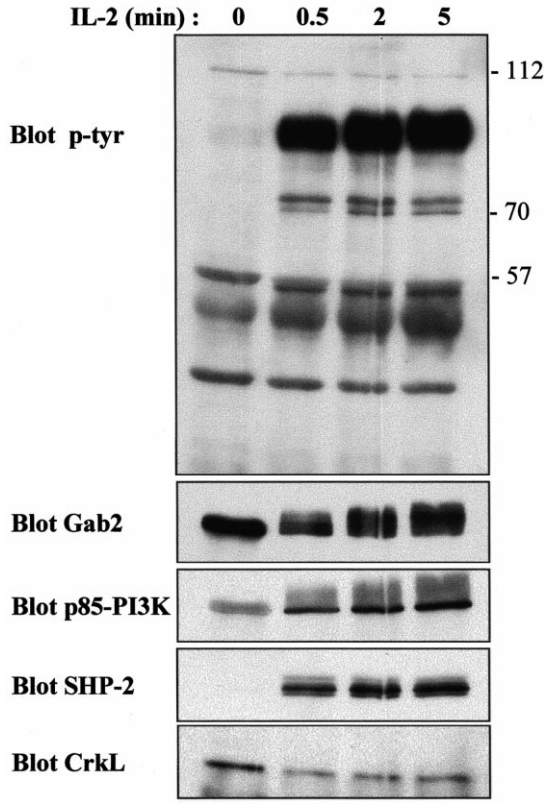

Fig. 1. Phosphorylation of Gab2 and protein interactions in IL-2-stimulated Kit 225 cells. Cell lysates from starved or IL-2-stimulated Kit 225 cells, were immunoprecipitated with anti-Gab2 antibodies and protein G Sepharose beads. Following SDS–PAGE separation, the precipitated material was analyzed by Western blotting with the indicated antibodies.

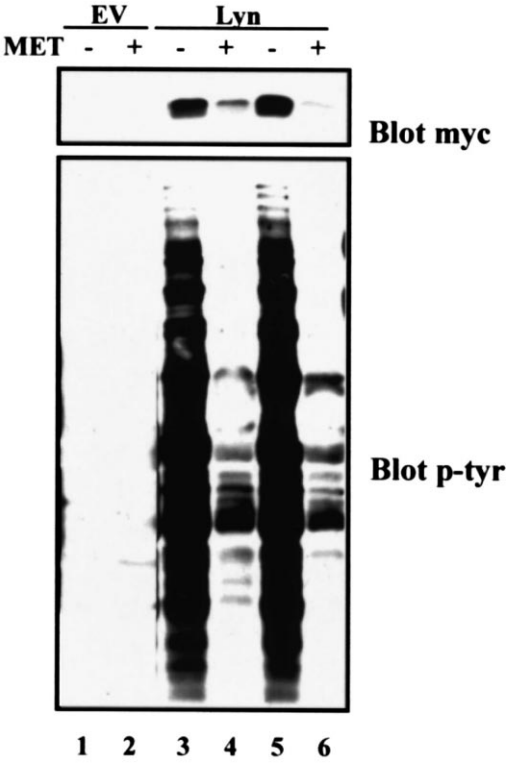

Fig. 2. Regulated expression of catalytic Lyn in yeast. Yeast diploids were obtained by mating W303 transformed with empty pMET416 vector (EV) or pMET416-Lyn (Lyn) with TAT7 transformed with empty pGad and either empty pLex (lanes 1,2), pLex-p85 (lanes 3,4) or pLex-SHP-2 (lanes 5,6). Cells were grown overnight in selective medium plus/minus methionine (MET) and protein extracts analyzed by Western blotting with 9E10 anti-myc or 4G10 anti-phosphotyrosine antibodies.

This modified two-hybrid system was then used to analyze the interactions of full length Gab2 with a variety of SH2 domains that are relevant to signaling by the interleukin 2 receptor in human lymphocytes [13]. As shown in Fig. 3, expression of the Lyn kinase did not affect a positive control interaction between Ras(V12) and Raf. Expression of the kinase also yielded no background with any of the pLex-SH2 domains when tested against empty pGad. Interaction of Gab2 was clearly detectable with the SH2 domains of CrkL and of SHP-2 in the presence of active Lyn. For reasons that have not yet been clarified, a constitutive interaction occurred between Gab2 and the SH2 domains of p85 PI3K, even in the presence of empty pMET416. Quantitation in a liquid β-galactosidase assay of the interaction occurring when Lyn was expressed however revealed a tyrosine phosphorylation-dependent interaction above background (data not shown). Inversing the constructs, i.e. cloning the p85-SH2s into pGad and Gab2 into pLex eliminated the background and clearly demonstrated that an interaction did take place between Gab2 and the p85 SH2s depending on Lyn expression (compare lines 7 and 9 in Fig. 3). Therefore, all subsequent studies of Gab2/p85 interactions were done in this orientation. However, incomplete repression of the kinase probably accounts for the interaction being still detectable in the presence of methionine. All positive interactions described above have been confirmed in a histidine prototrophy assay (not shown). Finally, Gab2 did not interact in this assay with the SH2 domains of Shc or of either STAT5 isoforms (a and b) that are known to be involved in interleukin-2 signaling [14–16].

3.2. Gab2 subdomains interacting with CrkL, SHP-2 and PI3K

SH2 domains bind to specific target sequences, depending on the nature of the residue located in position +3 relative to the phosphorylated tyrosine, that define consensus binding sites for a given SH2 [17]. Of practical interest, the putative binding sites for CrkL, PI3K and SHP-2 are ordered in three separate clusters on the Gab2 molecule. Indeed four consensus recognition sequences (YxxP) for the Crk SH2 are located at positions 249, 266, 324, and 409, three recognition sequences (YxxM) for p85 SH2s are located at positions 452, 476 and 584 and two recognition sequences (YxxV/L) for SHP-2 SH2s are located at positions 614 and 643. We therefore constructed, and analyzed for their interactions, Gab2 subdomains comprising aa 233–432, 445–611 and 601–676. The results of these experiments (shown in Fig. 4A) clearly evidenced the interactions with the three SH2 constructs and confirmed that they were restricted to the predicted subregions of Gab2. In addition, quantitation of these interactions in a liquid β-galactosidase assay indicated that SHP-2 associated with Gab2 in a somewhat weaker manner than PI3K or CrkL did (Fig. 4B).

3.3. Identification of tyrosine residues involved in Gab2 interactions with PI3K, SHP-2 and CrkL SH2 domains

Having located the subregions of Gab2 that interact with each of the partner SH2 domains, we undertook to systematically mutate into phenylalanine every tyrosine residue that is

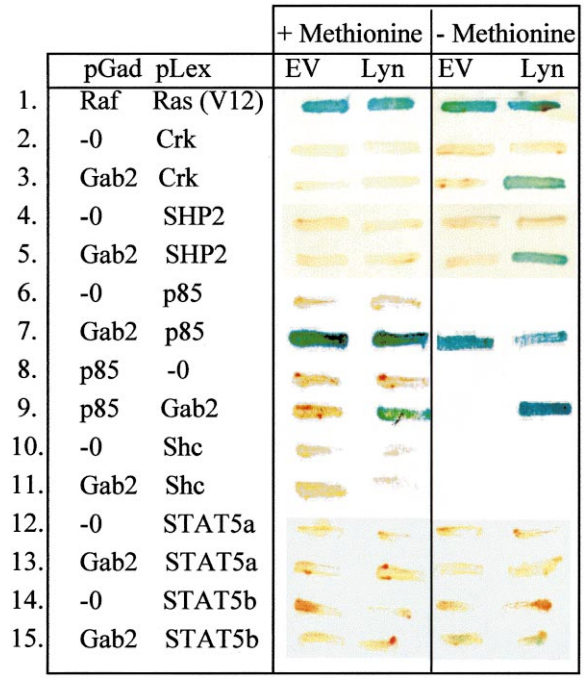

Fig. 3. Tyrosine phosphorylation-dependent interaction of Gab2 with the SH2 domains of CrkL, PI3K and SHP-2. Yeast diploids were obtained by mating W303 transformed with empty pMET416 vector (EV) or pMET416-Lyn (Lyn) with TAT7 transformed with the indicated constructs. Yeasts were grown overnight on filters layered over plates containing or not methionine. The filter β-galactosidase assays were performed as described in Section 2. The data shown are pooled from three experiments, each performed at least twice with identical results.

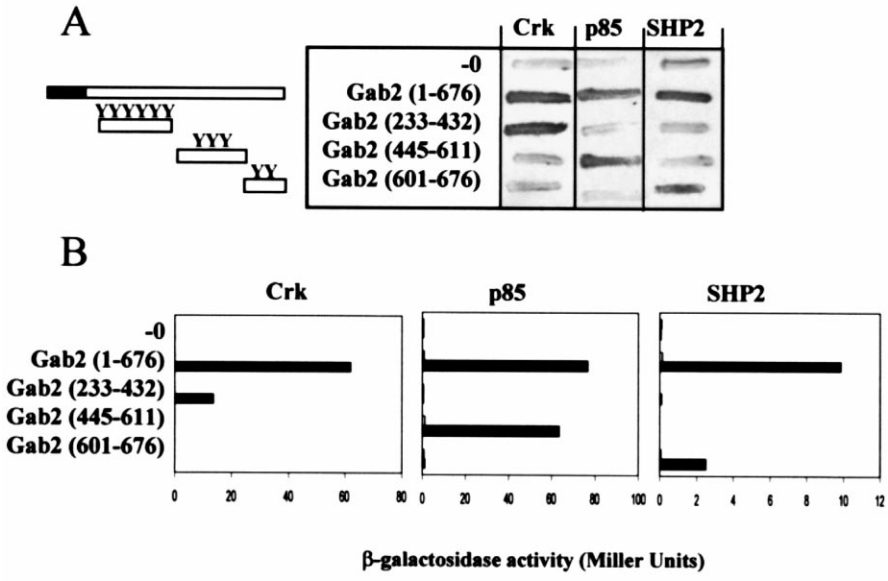

Fig. 4. Gab2 subdomains interacting with CrkL, SHP-2 and PI3K. A: schematic representation of Gab2 subregions, and filter β-galactosidase assay performed in the absence of methionine. B: Liquid β-galactosidase assay performed in the absence (filled bars) or in the presence (open bars) of methionine. The following combinations were used: the SH2 domains of SHP-2 or CrkL were inserted in pLex and tested against Gab2 inserted in pGad; the p85 SH2s were inserted in pGad and tested against pLex-Gab2.

potentially implicated in these interactions. This was performed by sequential mutagenesis on the three subregion-encoding plasmids, and the overall data for filter and liquid β-galactosidase assays are shown in Table 1.

3.3.1. Tyrosine residues involved in p85-PI3K recognition. The p85-SH2 interacting subregion of Gab2 (aa 445–611) contains three tyrosine residues at position 452, 476, 584 which are followed by a M in position +3. Mutating into phenylalanine all three tyrosines completely abrogated the interaction, and anyone of these tyrosine residues, when expressed alone, yielded significant β-galactosidase activity. Thus all three tyrosines predicted to bind p85 are indeed functional in this assay. Although differences in the level of β-galactosidase activity were reproducibly observed,

Table 1
Identification of tyrosine residues involved in Gab2 interactions

P97 mutants	Y residue(s) expressed	filter assay (−**Met**)	Liquid assay (Miller units)[a]	
			(− **Met**)	(**+Met**)
PI3K interaction[b]				
YYY	all	Blue	15.7	3.3
FFF	none	White		0.50.8
YFF	452	Blue	13.9	2.0
F**Y**F	476	Blue	25.7	2.0
FF**Y**	584	Blue	8.9	1.4
YYF	452, 476	Blue	24.3	2.5
F**YY**	476, 584	Blue	15.3	2.1
YF**Y**	452, 584	Blue	23.7	2.3
SHP-2 interaction[c]				
YY	both	Blue	2.2	0.1
FF	none	White		0.20.0
YF	614	Blue	3.2	0.1
F**Y**	643	White	0.1	0.1
CrkL interaction[d]				
YYYYYY	all	Blue	14.8	0.8
FYFF**Y**	293, 411	Blue	37.8	1.0
FFFFFF	none	White	0.6	0.5
YFFFFF	249	White	0.8	0.5
FYFFFF	266	Blue	64.4	1.0
FF**Y**FFF	293	Blue	35.7	1.0
FFF**Y**FF	324	White	0.7	0.5
FFFF**Y**F	409	White	0.7	0.5
FFFFF**Y**	411	White	0.7	0.5
YFFYYY	249, 324, 409, 411	White	0.7	0.5

[a]Data shown are the mean of two independent determinations.
[b]Assayed with pLex-Gab2(445–611) versus pGad-p85-(N+C)SH2.
[c]Assayed with pGad-Gab2(601–676) versus pLex-SHP-2-(N+C)SH2.
[d]Assayed with pGad-Gab2(233–432) versus pLex–CrkL–SH2.

we could not establish a clear hierarchy between individual tyrosine residues (or pairs) regarding their relative importance in the interaction.

3.3.2. Tyrosine residues involved in SHP-2 interaction. The SH2 domains of SHP-2 have been described to preferentially recognize pY followed either by V or by L in position +3 [17]. Thus the two tyrosine residues at the C-terminal end of Gab2 (Y_{614}LAL and Y_{643}VQV) represent potential binding sites for SHP-2. The results shown in Table 1 however demonstrate that Y_{614} alone mediates this interaction. Indeed, mutation of Y_{643} had no detectable effect on the interaction, yielding almost identical values as the wild type in the assay, whereas mutation of Y_{614} completely abrogated the interaction.

3.3.3. Tyrosine residues involved in CrkL SH2 recognition. Within the CrkL-binding subdomain, which contains six Y residues, we initially mutated the four Y residues (at positions 249, 266, 324, and 409) that are followed by P in position +3 and may thus represent putative binding sites for CrkL SH2. Unexpectedly, a strong interaction was still detected, as measured in the liquid assay for β-galactosidase activity with this FFYFFY mutant (Table 1). Mutating to F the remaining two Y (Y_{293}TFK and Y_{411}PQR) in this region abolished the interaction. To identify which residues was (were) involved, single Y were reintroduced within the context of the remaining five being F. As evidenced in Table 1 only residues Y_{266} and Y_{293}, when expressed alone mediated significant binding. These two residues accounted for all the interaction between CrkL and Gab2, since the YFFYYY mutant was negative in this assay. Thus out of the four YxxP sequences, only one (Y_{266}DLP) appears to be involved in binding CrkL SH2, together with Y_{293} whose environment (YTFK) does not fit the consensus but which is also clearly recognized by CrkL SH2.

3.4. In vitro binding of CrkL with Gab2-derived peptides

Since the interaction of CrkL with Y_{293} was not predicted based on known consensus, we sought to confirm it by a biochemical approach. 15-amino acid long peptides centered on Y_{266} and on Y_{293} were synthesized, with the tyrosine phosphorylated or not, and used to study association of either natural CrkL, as provided in Kit 225 cell lysates, or recombinant CrkL SH2 domain produced as a GST fusion protein. As seen in Fig. 5, both the GST–CrkL–SH2 and the endogenous CrkL associated efficiently with the Y_{293} phosphopeptide and with the Y_{266} phosphopeptide. This interaction was indeed found to depend upon peptide 293 being tyrosine-phosphorylated, and its specificity further confirmed in that none of the peptides used in these experiments interacted with SHP-2 (data not shown).

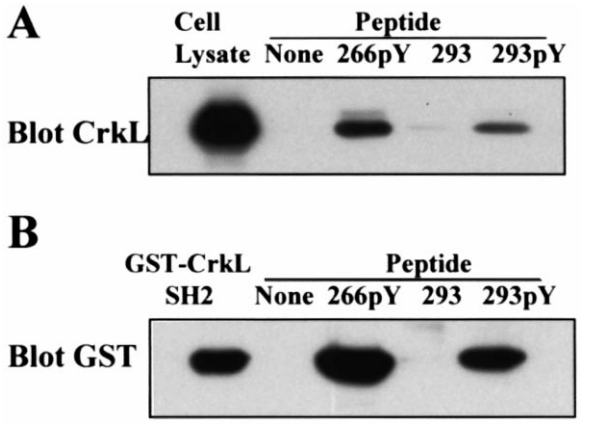

Fig. 5. In vitro interactions of CrkL with Gab2-derived phosphopeptides. Biotinylated synthetic phosphopeptides centered on human Gab2 Y_{266} (266pY, sequence: EFRDST[pY]DLPRSLAS) and on Y_{293} (293pY, sequence: TDNEDV[pY]TFKTPSNT) were coupled to streptavidin beads and incubated with either Kit 225 cell lysates (A) or purified GST–CrkL–SH2 fusion protein (B). Uncoupled beads and non-phosphorylated Y293 peptide (293) were used as controls. Western blots were performed with a CrkL antiserum (A) or with anti-GST antibodies (B).

4. Discussion

Interactions of SH2 domains with phosphorylated tyrosines are critical for signal transduction from many membrane receptors [18,19]. In recent years it has been found that adapter proteins of the IRS/Gab family bind multiple SH2 domain-containing molecules and provide both time and space coordination to multiple signaling pathways. Gab2 contains 19 tyrosine residues, and although the identification of which residues are actually phosphorylated in growth factor-stimulated cells has not been reported, 11 of these residues are preceded by acidic (E or D) amino acids and are thus potential phosphorylation sites for Src family kinases [20]. The various stimuli that lead to Gab2 phosphorylation have all in common that they activate members of the Src family. In preliminary experiments we found that the p56lck tyrosine kinase did not perform well in this yeast two-hybrid system. We therefore decided to use Lyn, another Src kinase involved in signaling in lymphoid cells, in our modification of the two-hybrid system to identify tyrosine residues which are critical for the association of human Gab2 with p85-PI3K, SHP2 and CrkL. Our use of a methionine regulatable promoter to express the catalytic domain of Lyn provided internal control, formally establishing that the interactions we describe here depend on phosphorylation. We first identified three subregions of Gab2 that interact with p85, SHP2 and CrkL, whereas the PH domain-containing N-terminal region (aa 1–276) did not associate with any of the SH2 domains studied here (not shown).

Shc is an essential adapter that recruits Gab2 to phosphorylated receptors. In most cases, Shc recognizes phosphorylated receptors through its PTB domain, and is then phosphorylated, thus providing a docking site for Grb2 to which Gab2 is bound through SH3/polyproline interactions [16,21,22]. Although it has previously been suggested that Shc could associate with Gab2, depending on its SH2 domain [23], our experiments did not evidence such an interaction, indicating that it is most likely indirect, or of too low an affinity to be detected in this yeast two-hybrid assay. Other SH2 domains that we found not to interact with Gab2 in this assay are those of STAT5, which are not co-immunoprecipitated with Gab2 in interleukin-2 stimulated lymphocytes (not shown).

Association of Gab2 with p85-PI3K was straightforward as the three Y residues which, based on sequence environment, are predicted to associate with p85-PI3K SH2 were found to yield strong interactions in this assay. In contrast, although two Y residues of Gab2 (i.e. Y_{614} and Y_{643}) were predicted to mediate interaction with SHP2 SH2, mutating Y_{614} alone completely abrogated the interaction. A similar observation has been made for Gab1, which, of the two homologous Y

residues (627 and 659) appears to only use the former to associate with SHP-2 [9]. However, it cannot be totally excluded that Y_{643} could be recognized with too low an affinity to give a detectable interaction in the yeast two-hybrid. Indeed, a model has been proposed whereby the SHP2 N-terminal SH2 domain binds phosphotyrosine with a lower affinity than the C-terminal SH2 [24]. Thus, binding of the tandem SH2 to a bisphosphoryl ligand would occur sequentially, with initial high energy binding on one site by the C-SH2 allowing the N-SH2 to then engage the second site. Our results suggest that Gab2 Y_{614} might be a preferential binding site for the C-SH2, and experiments aimed at verifying this hypothesis are currently in progress. Others have already reported that mutating the three YxxM in the PI3K-binding region of murine Gab2 abrogates its binding to PI3K, and that mutating the two C-terminal tyrosine residues abrogated binding to SHP-2, and demonstrated that these interactions are critical for downstream signaling events in cytokine response [25,26].

In contrast, the functional significance of the interaction between Gab2 and CrkL has not yet been investigated. CrkL is an adapter protein whose main partners are on one hand the ras exchange factors C3G and SOS and, on the other hand, the cytoskeleton regulatory proteins p125Fak and paxilin [27–29]. Through these multiple interactions, CrkL may thus be involved in the regulation of the ras pathway and control cytoskeletal functions in response to growth factors but also upon integrin activation [30]. In addition, CrkL itself becomes tyrosine-phosphorylated in response to growth factor stimulation [31]. CrkL contains two SH3 and one SH2 domains. Whether the constitutive interaction between Gab2 and CrkL that is observed in unstimulated cells (Fig. 1) is mediated by the CrkL SH3 domains recognizing Gab2 polyproline motifs has not been investigated in this study which was focused on SH2–phosphotyrosine interactions. We demonstrate here that the CrkL SH2 domain can indeed associate with Gab2 in the yeast assay, and can recognize Gab2-derived phosphopeptides. An unexpected finding in this report is that out of the four YxxP sequences which are present in the CrkL-binding region of Gab2, only tyrosine Y_{266} mediates significant interaction. Of note, Gab2 contains an additional Y_{563}CRP, within the PI3K-binding region, that does not appear to be recognized by CrkL. In contrast, CrkL SH2 does interact with Y_{293}TFK whose sequence environment does not fit the consensus. It may be of interest that Gab1 does not contain an equivalent YTFK sequence, and that all of its interaction with CrkL appears to depend upon typical YxxP motifs [29].

Our results identifying the binding sites for these critical partners of Gab2, provide the basis for introducing mutations in Gab2 to specifically abrogate these interactions and further analyze their functional significance in cells. Finally, the modification of the two-hybrid system described above could be used to screen cDNA libraries for identifying new targets for specific SH2 domains.

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