Interaction of p85 subunit of PI 3-kinase with insulin and IGF-1 receptors analysed by using the two-hybrid system

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Abstract **Interaction of the p85 subunit of PI 3-kinase with the insulin receptor (IR) and the IGF-1 receptor (IGF-1R) was investigated using the two-hybrid system by assessing for *his3* and *lacZ* activation in *S. cerevisiae*. The experiments were performed with the cytoplasmic β domain (wild type or mutated) of IR and IGF-1R and p85 or its subdomains (N + C-SH2, N-SH2, C-SH2, SH3 + N-SH2). The results of *his3* activation indicated that p85, N + C-SH2 and C-SH2 interact with both IRβ and IGF-1Rβ, whereas N-SH2 and SH3 + N-SH2 interact only with IRβ. Interaction of p85 and N + C-SH2 with IRβ(ΔC-43) or IGF-1Rβ(ΔC-43) in which the C-terminal 43 amino acids (including the YXXM motif) were deleted, persisted. The internal binding site thus revealed was not altered by further mutating Y^{960}/F for IR or Y^{950}/F for IGF-1R. Activation of *lacZ* upon interaction of p85 with IRβ(ΔC-43) was 4-fold less as compared to IRβ. This activation with p85 and IGF-1Rβ was 4-fold less as compared to IRβ and was somewhat increased (2-fold) for IGF-1Rβ(ΔC-43). Thus, the C-terminal domain in IGF-1R appears to exert a negative control on binding of p85 thereby providing a possible regulatory mechanism for direct activation of the PI 3-kinase pathway.**

Key words: Insulin receptor; IGF-1 receptor; Signal transduction; Phosphatidylinositol 3-kinase; Two-hybrid system

1. Introduction

The insulin receptor (IR) and IGF-1 receptor (IGF-1R) are very homologous in their primary as well as their heterodimeric $\alpha_2\beta_2$ structure and belong to the family of membrane receptors with tyrosine kinase activity [1]. It is well established that receptor autophosphorylation upon ligand binding and activation of the tyrosine kinase domain are required for most, if not all, of the biological effects of insulin and IGF-1. The molecular basis of the differential signalling by these two receptors leading to common as well as specific biological effects is still not entirely understood. One of the mechanisms of signalling by IR and IGF-1R consists in the phosphorylation by the activated receptor tyrosine kinase of intermediate docking proteins such as insulin receptor substrate-1 (IRS-1) and SHC which subsequently bind various Src homology 2 (SH2)-domain containing effector proteins that activate different signalling pathways [2,3]. For instance, the regulatory p85 subunit of PI 3-kinase binds to phosphotyrosines at YXXM motifs [4] in IRS-1 and thereby activates the catalytic p110 subunit [2]. Surprisingly, inactivation of the IRS-1 gene in the mouse by using the homologous recombination approach did not result in any dramatic pathological phenotype, indicating the possible existance of alternative signalling pathways [5,6].

A number of studies performed mainly with IR have provided evidence that some effector proteins such as the p85 subunit of PI 3-kinase, GTPase activating protein (GAP) and SYP (SH2-domain-containing tyrosine phosphatase) could directly bind via their SH2 domains to phosphotyrosines of the cytoplasmic domain of the receptor [7–11] and this could represent an alternative mechanism by which some signalling pathways could be activated or regulated. In IR, the binding site for p85 and SYP has been mapped to the C-terminal Y^{1322} and for GAP to the NPXY motif at Y^{960} [12]. Interaction of these proteins with IGF-1R has not been examined in detail and only weak direct interaction of p85 with IGF-1R has been reported [13,14].

Recently, the two-hybrid approach [15,16] was applied to investigate the interaction of IRS-1 with the cytoplasmic β domain of IR [17]. In the work reported here, we have used the two-hybrid system to perform a comparative analysis of direct interaction of p85 with the cytoplasmic β domain of IR and IGF-1R.

2. Experimental

2.1. Plasmid construction

All *Escherichia coli* (strain HB 101) and DNA manipulations were performed essentially as described [18]. Enzymes used for cloning were from Biolabs and Pfu DNA polymerase from Gibco-BRL. Primers used for PCR were purchased from Genosys and all 5'-deoxynucleotide triphosphates were from Boehringer. Plasmids carrying cloned full-length cDNAs encoding IR [19,20] and p85 [21–23] were generously provided by A. Ullrich and cloned full-length cDNA for IGF-1R [24,25] was obtained from P. De Meyts. The plasmids used for hybrid gene constructs were pBTM116 ([26]; carrying *trp1*) and pGAD GH ([27]; carrying *leu2*), encoding the DNA binding domain of LEXA and GAL4 activation domain (GAD), respectively.

The DNAs corresponding to the cytoplasmic IRβ and IGF-1Rβ were PCR amplified using cloned cDNAs with different sets of the following primers (5' to 3'): CGCGAATTCAGAAAGAGGCAGCCAGATGG (R1), CGCGGATCCTTAGGAAGGATTGGACCGAG (R2), CGCGAATTCAGAAAGAGAAATAACAGCAG (R3), CGCGGATCCAAGGATCAGCAGGT (R4), CGCGGATCCTTACTCCTCCCTCTGACAGTGCG (R5), CGCGGATCCTTACTTGTGTCCTGAGTGTCTGT (R6),CGCGAATTCAGAAAGAGGCAGCCAGATGGGCCGCTGGGACCGCTTTACGCTTCTTCAAACCCTGAGTTTCTCAG (R7), CGCGAATTCAGAAAGAGAAATAACAGCAGGCTGGGGAATGGAGTGCTGTATGCCTCTGTGAACCCGGAGTTCTTCAG (R8), CGCGGATCCTTAGGTCGAAGACTGGGGCAGCG (R9). The *Eco*RI and *Bam*HI sites are underlined. The PCR products were cloned into pBTM116 using *Eco*RI/*Bam*HI sites. The plasmids obtained by cloning the DNA fragments amplified with the primer sets R1/R2, R3/R4, R1/R5, R3/R6, R7/R2, R8/R4 and R3/R9 encode LEXA hybrid proteins with IRβ, IGF-1Rβ, IRβ(ΔC-43), IGF-

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Abbreviations: IGF-1, insulin-like growth factor-1; PI 3-kinase, phosphatidylinositol 3-kinase; aa, amino acid; C, Cys; H, His; M, Met; N, Asn; P, Pro; R, Arg; T, Thr; Y, Tyr; X, any of the 20 aa.

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1Rβ(ΔC-43), IRβ(ΔC-43 + Y^{960}/F), IGF-1Rβ(ΔC-43 + Y^{950}/F) and IGF-1Rβ(C^{1337}/Δ), respectively. The various forms of the cytoplasmic β domain used are described in section 3.1.

Several subdomains of p85 were PCR amplified using cloned cDNA with different sets of the following primers (5′ to 3′): CGCTCTAGA-CGGTATGAATAACAATATGTC (P1), CGCGAATTCTTAATCT-TCTTTGACAACTTGAT (P2), CGCTCTAGACGAAGATTTGCC-CCATCATGA (P3), CGCGAATTCTCATCGCCTCTGCTGTG-CAT (P4), CGCTCTAGACAGTGCTGAGGGGTACCAGTA (P5), CGCGAATTCTTACCGGGGCTTTGGTGTGGGAG (P6). The *Xba*I and *Eco*RI sites are underlined. The PCR products cut with *Xba*I/*Eco*RI were cloned into pGAD GH cut with *Spe*I/*Eco*RI. The plasmids obtained by cloning the DNA fragments amplified with the primer sets P1/P2, P3/P4, P1/P4, P5/P6 and P5/P2 encode GAD hybrid proteins with N-SH2 (aa 321–440), C-SH2 (aa 614–725), N + C-SH2 (aa 321–725), SH3 (aa 2–90) and SH3 + N-SH2 (aa 2–440), respectively. To engineer a plasmid expressing GAD fusion protein with full-length p85, the *Sac*I/*Apa*I fragment from pRK-p85 was cloned in the plasmid encoding GAD hybrid protein with SH3 + N-SH2 cut with *Sac*I/*Apa*I.

All PCR were performed using Pfu DNA polymerase according to the supplier under the following conditions. After an initial denaturing step at 95°C for 5 min, 30 cycles were performed each consisting of 1 min at 95°C, 1 min at 55°C and 2 min at 72°C; the final cycle was followed by a further 5 min incubation at 72°C.

The DNAs for all the recombinant plasmids (two independent clones for each construct) were amplified and were used to transform yeast cells.

2.2. Transformation and growth of yeast

Yeast strain L40 (*trp1*⁻, *leu2*⁻, *his3*⁻, *lexAop-his3*, *lexAop-lacZ*) was transformed by the lithium acetate method [28] using different combinations of plasmids. The transformants were selected on 2% glucose minimal agar media lacking Trp and Leu.

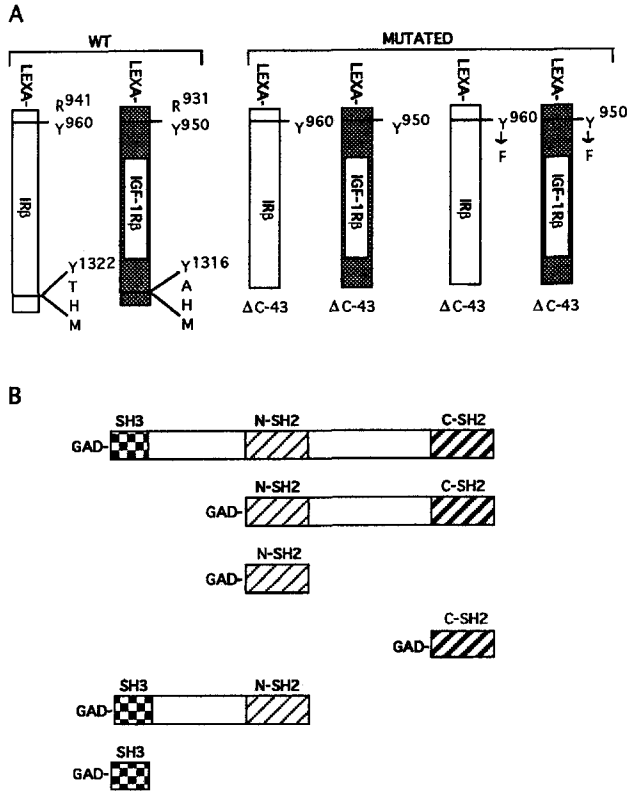

Fig. 1. Structure of LEXA hybrid proteins with wild type (wt) or mutated cytoplasmic β domains of IR and IGF-1R (A) and of GAD hybrid proteins with p85 or its subdomains (B).

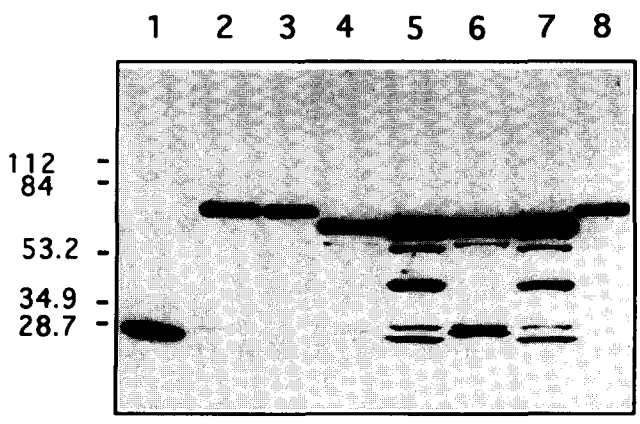

Fig. 2. Western immunoblot analysis of protein extracts from yeast strains transformed with plasmids encoding LEXA or its hybrid proteins. Lane 1: LEXA, Lane 2: LEXA-IRβ, Lane 3: LEXA-IGF-1Rβ, Lane 4: LEXA-IRβ(ΔC-43), Lane 5: LEXA-IGF-1Rβ(ΔC-43), Lane 6: LEXA-IRβ(ΔC-43 + Y^{960}/F), Lane 7: LEXA-IGF-1Rβ(ΔC-43 + Y^{950}/F), Lane 8: LEXA-IGF-1Rβ(C^{1337}/Δ). The size markers (in kDa) were from Bio-Rad.

2.3. Western immunoblot analysis of hybrid proteins

Ten milliliters of selective medium (lacking Trp and Leu) with 2% glucose were seeded with overnight cultures from colonies of selected yeast strains to an optical density at 600 nm of 0.2 and incubated at 30°C until the optical density reached 0.5. The cells were pelleted, resuspended in 400 μl of sodium-dodecyl-sulfate (SDS) sample buffer and vortexed with glass beads. After centrifugation, the supernanant was collected, boiled and 10 μl were subjected to SDS-polyacrylamide gel electrophoresis [29]. The proteins were blotted to nitrocellulose [30] and LEXA hybrid proteins were detected by using an antibody against the DNA binding domain of LEXA (obtained from P. Moreau) and the ECL detection system (Amersham).

2.4. β-Galactosidase assays

The solution β-galactosidase assays were performed using o-nitrophenyl β-D-galactopyranoside (ONPG) as substrate and the units of β-galactosidase calculated as described [31]. One unit of β-galactosidase corresponds to $OD420 \times 1000/t \times v \times p$, where t represents minutes of the reaction, v is milliliters of supernatant used, and p is protein concentration in mg/ml.

3. Results and discussion

The two-hybrid system represents a genetic approach to characterize protein–protein interactions in yeast [15,16]. Basically, the two proteins are expressed in yeast as two hybrids: one with the DNA binding domain of a transcription factor (i.e. LEXA) and other with the activation domain of a transcription factor (i.e. GAL4). The yeast strains used possess *his3* and/or *lacZ* carrying upstream activating sequences that allow transcriptional activation of these genes if a functional transcription factor is reconstituted upon protein–protein interaction between the two hybrids. This approach has been applied here to analyse the interaction of the p85 subunit of PI 3-kinase with IR and IGF-1R.

3.1. Engineering and expression of hybrid proteins

The DNA binding domain of LEXA was fused to the cytoplasmic β domain starting from the first aa after the transmembrane domain, i.e. R^{941} for IR and R^{931} for IGF-1R. In the study of the interaction of the cytoplasmic β domain of IR with IRS-1 using the two-hybrid approach, it was shown that the β domain

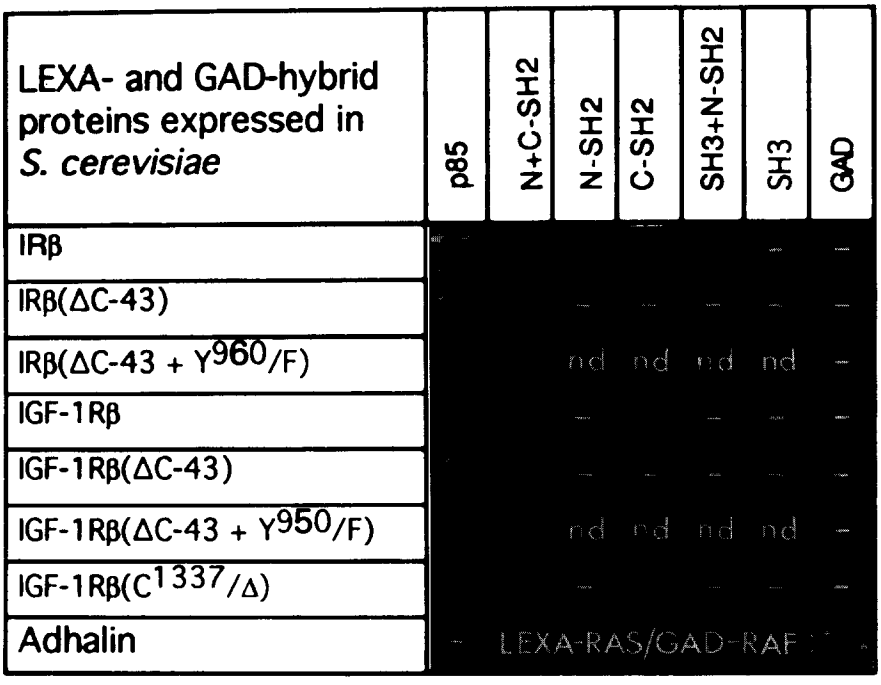

Fig. 3. Growth of yeast strains resulting from *his3* activation. Yeast strains cotransformed with plasmids encoding various hybrid proteins grown on 2% glucose minimal agar medium lacking Trp and Leu were tested for their ability to grow on the same medium also lacking His.

is phosphorylated in such a hybrid in the yeast [17]. Hybrids of LEXA with $\beta(\Delta$C-43) in which C-terminal 43 aa were deleted or with $\beta(\Delta$C-43) further containing Y^{960}/F mutation for IR and Y^{950}/F for IGF-1R were also engineered (Fig. 1A). In the case of IGF-1R, $\beta(C^{1337}/\Delta)$ in which the terminal C^{1337} was deleted was also fused to LEXA. The structure of various GAD hybrid proteins with p85 or its subdomains is shown in Fig. 1B.

Western immunoblot analysis of protein extracts for yeast strains transfected with plasmids encoding LEXA or its hybrid proteins showed that all the proteins were expressed (Fig. 2). We assume that GAD hybrid proteins are also expressed even though attempts to detect these proteins were unsuccessful due to high background and unspecific reactions obtained with the antibodies against GAD which we used.

3.2. Interaction of p85 with IR

Yeast strains coexpressing LEXA-IRβ and GAD hybrid proteins with p85, N + C-SH2, N-SH2, C-SH2 or SH3 + N-SH2 initially selected on minimal medium lacking Trp and Leu were all able to grow on the same medium also lacking His (Fig. 3). Activation of *his3* in these transformed strains provides genetic evidence that p85 or its subdomains can directly interact with IRβ. The growth of yeast strains resulting from such interactions is comparable to the growth of a yeast strain coexpressing hybrid proteins of LEXA and GAD with RAS and RAF, respectively (Fig. 3), two proteins that are known to interact strongly [26]. The SH3 domain of p85 did not interact with IRβ. Finally, no *his3* activation was obtained when GAD-p85 was coexpressed with hybrid of LEXA with Adhalin ([32]; obtained from J. Chelly), a protein unrelated to insulin signalling.

It is known that the SH2 domains of p85 bind to phosphotyrosines at YXXM motifs [4]. Recently, the binding site of p85 in IR was mapped to the YTHM motif at Y^{1322} in the C-terminal domain on the basis of (i) in vitro precipitation of partially purified IR but not of IR deleted for the C-terminal 43 aa by glutathione-S-transferase fusion proteins containing p85 or its subdomains and (ii) the ability of various pY peptides to inhibit this precipitation [7,12]. The ability of p85 or its subdomains to bind to LEXA-IR$\beta(\Delta$C-43) was thus examined (Fig. 3). From *his3* activation, it can be concluded that the

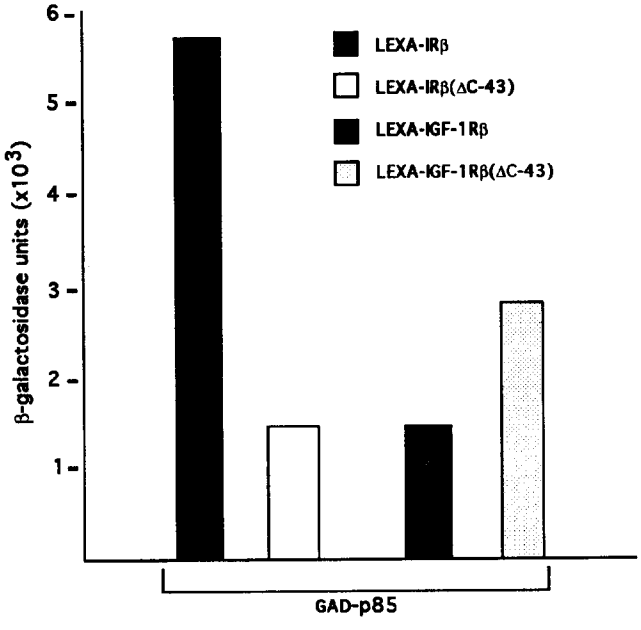

Fig. 4. β-Galactosidase activity in protein extracts from yeast strains transformed with plasmids encoding GAD-p85 and LEXA hybrid proteins as indicated. The mean value of at least three independent experiments is presented. In each case, the values differed less than 10% from one experiment to another. A blank value (~10 units) obtained with an extract from yeast strain expressing GAD/LEXA had been substracted.

interaction of N-SH2, C-SH2 and SH3 + N-SH2 with IRβ is indeed abolished upon deleting the C-terminal 43 aa. However, p85 and N + C-SH2 continue to interact with IRβ(ΔC-43) thus revealing the presence of a second internal binding site that is not capable of binding any of the single SH2 domains used here.

In a previous study examining direct interaction of p85 with IR using an immunoprecipitation/cross-linking approach, Y^{960} was implicated as a possible binding site for p85 [8]. We therefore introduced Y^{960}/F mutation in IRβ(ΔC-43) and showed that this mutation did not have any effect on *his3* activation using p85 or N + C-SH2 (Fig. 3).

Interaction of p85 with IRβ as well as with IRβ(ΔC-43) was further examined by analysing activation of *lacZ* reporter (Fig. 4). Quantitative analysis showed that activation of *lacZ* upon interaction of p85 with IRβ(ΔC-43) was decreased 4-fold as compared to IRβ.

3.3. Interaction of p85 with IGF-1R

Activation of *his3* was observed in yeast strains coexpressing LEXA-IGF-1Rβ and GAD hybrid proteins with p85, N + C-SH2 and C-SH2, indicating direct interaction between p85 and IGF-1Rβ (Fig 3). In contrast to what was observed with IRβ, no interaction between N-SH2 or SH3 + N-SH2 and IGF-1Rβ was detected.

The cytoplasmic β domains of IR and IGF-1R are very similar [24] and a YAHM motif can also be found at Y^{1316} at the C-terminal part of IGF-1R. The ability of p85 or its subdomains to activate *his3* upon binding to IGF-1Rβ(ΔC-43) indicates that as for the IRβ(ΔC-43), p85 and N + C-SH2 continue to interact with IGF-1Rβ(ΔC-43) whereas the binding of C-SH2 was abolished (Fig. 3). This internal binding was not affected when Y^{950}/F mutation was introduced in IGF-1Rβ(ΔC-43).

Interestingly, growth of yeast strains upon *his3* activation resulting from interaction between p85 and IGF-1Rβ(ΔC-43) appeared to be higher as compared to IGF-1Rβ. This observation was confirmed by analysing *lacZ* activation that was 2-fold higher with p85 and IGF-1Rβ(ΔC-43) as compared to IGF-1Rβ (Fig. 4). Since IGF-1R contains a C residue at the C-terminus that is not present in IR, one could imagine that C^{1337} might be involved in a disulfide bond that could somehow hinder binding of p85 to IGF-1R. This was not the case since p85 or its subdomains interact equally well with IGF-1Rβ(C^{1337}/Δ) and IGF-1Rβ (Fig. 3).

3.4. Differential interaction of p85 with IR and IGF-1R

A comparative analysis of the interaction of p85 with IRβ and IGF-1Rβ using the two-hybrid system indicates that the interaction of p85 with IR is much stronger as compared to IGF-1R. These results are compatible with previous immunoprecipitation studies that reported only weak interaction between p85 and IGF-1R [13,14]. Deletion of the C-terminal 43 aa in IRβ lead to a decreased interaction with p85 whereas in the case of IGF-1Rβ, this deletion rather resulted in a slight increase in the interaction with p85.

In conclusion, it is tempting to speculate that the C-terminal domain in IGF-1R might exert a negative control on the binding of p85 thereby providing a means of regulating the activation of the PI 3-kinase pathway upon its interaction with IGF-1R. Such control might represent one of the mechanisms which could contribute to achieving specificity in metabolic versus mitogenic signalling by these two receptors. In addition, IR and IGF-1R appear to contain two sites for the binding of p85: one site comprising the C-terminal YXXM motif and a second internal site which remains to be characterized.

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