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Mutational analysis of the interaction between insulin receptor and IGF-I receptor with c-Crk and Crk-L in a yeast two-hybrid system☆,☆☆

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Abstract

The SH2/SH3 adapter proteins of the Crk family are potent signal transducers after receptor tyrosine kinase stimulation with insulin or IGF-1. We have employed a yeast two-hybrid approach and mutational analysis to dissect the capabilities of the insulin receptor and the IGF-I receptor to directly associate with Crk isoforms. Insulin receptor stably recruits full length Crk by association with its SH2 domain in an auto-phosphorylation dependent manner. In contrast, interaction of the IGF-I receptor with the Crk-IISH2 domain was only detectable when Crk-II was truncated in its C-terminal part, indicating the transient nature of this interaction. From these data it can be concluded that members of the insulin receptor family activate Crk proteins in a differential manner.

Keywords: Insulin receptor; Type 1 insulin-like growth factor receptor; Signal transduction; c-Crk; Crk-L; Tyrosine phosphorylation; Yeast two-hybrid

The crk gene family consists of cellular homologues of the viral oncogene v-crk [1–3]. The gene products Crk-L, Crk-II and its alternatively spliced isoform Crk-I have been implicated in a number of biological processes such as cell differentiation, cell migration, T-cell anergy, and signal transduction of the CML-causing Bcr-Abl oncoprotein (reviewed in [4,5]). Crk proteins possess no enzymatic activity but exert their biological function by integrating various signalling pathways through their modular structure. They are adapter proteins consisting of a N-terminal Src homology domain (SH2) followed by two SH3 domains [referred to as SH3(N) and SH3(C) herein]. The SH3 domains often mediate interaction with effector molecules downstream of Crk that contain proline rich motif(s) conforming to the consensus sequence Pro-x-x-Pro. Most currently known SH3 mediated associations of c-Crk/Crk-L are mediated through the N-terminal SH3 domain. Prominent examples are the guanine nucleotide exchange factor (GEF) C3G [6], the regulatory subunit of the PI3-kinase p85 [7], and the non-receptor tyrosine kinase c-Abl [8,9]. The consensus high affinity-binding motif for the SH2 domain of c-Crk and Crk-L is pTyr-x-x-Pro [10]. Such motifs were found and proven to act as docking sites for the CrkSH2 domain in a variety of signalling molecules involved in diverse signal transduction processes. For instance, activation, i.e., tyrosine phosphorylation, of p130Cas and paxillin within focal adhesion contact sites leads to the recruitment of Crk proteins (among others) finally triggering the reorganization of the cytoskeleton and affecting cell motility and

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☆☆ *Abbreviations:* InsR, insulin receptor; IGF-IR, type I insulin-like growth factor receptor; IRS, insulin receptor substrate.
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morphology [11,12]. As an additional point of control growth factors can modify the signalling from extracellular matrix (ECM) or cell–cell contacts to the focal adhesion multi-protein complexes. c-Crk/Crk-L may thereby serve as one of the integration sites. One group of growth factors transmits the signal to Crk through activation of receptor tyrosine kinases (RTKs) followed by direct recruitment of Crk to phosphotyrosine residues of the receptors or by receptor catalysed phosphorylation of docking or adapter proteins, such as Grb2, Gab1, IRS-1 or IRS-4, and subsequent association with the CrkSH2 domain (reviewed in [5]). For some of the RTKs direct interaction with members of the Crk family has been suggested. In addition to the PDGF α- and β-receptors [13], the EGF receptor [14], the FGFR-1 [15], the proto-oncoprotein c-Ret and a mutated variant of it [16], the receptor for the stem cell factor c-Kit [17], and the Eph-family receptor EphB3 [18] assembly of type 1 insulin-like growth factor receptor (IGF-IR)–Crk complexes have been shown in vitro [19]. IGF-IR and insulin receptor belong to the insulin receptor family that in addition includes the ''orphan'' receptor insulin receptor-related receptor (IRR). Basically, although insulin receptor and IGF-I receptor share a high similarity in sequence and structure they elicit different cellular effects after activation by insulin or insulin-like growth factors, respectively. The IGF-I receptor preferentially controls developmental and growth processes whereas the insulin receptor is a major player in maintenance of metabolic homeostasis. This can be achieved by differential spatio-temporal availability of stimuli, signalling molecules, and effectors in combination with distinct capabilities of the receptors to recruit and/or activate signal transducers. Crk-II has been shown to be involved in the insulin stimulated GLUT4 translocation to the plasma membrane as a prerequisite for glucose uptake in adipocytes [20]. In order to activate the low molecular weight GTP-binding protein TC10 a Crk-II–C3G complex has to be assembled with a complex consisting of CAP, APS, and Cbl, where tyrosine phosphorylated Cbl is recognized by the Crk-IISH2 domain [21]. In addition, Crk-II becomes phosphorylated in response to insulin stimulation accompanied by a dissociation of Crk-II–C3G complexes in fibroblasts and CHO cells [22–24] followed by de-repression of Rap1 inhibitory function on the Raf1 kinase mediated MAP kinase activation. Similar to the insulin receptor, IGF-1 stimulated activation of the IGF-I receptor kinase leads to phosphorylation of Crk proteins in vivo [25–27]. In addition, in vitro experiments employing an activated IGF-I receptor as well as $(His)_6$-IGF-IR fusion peptides demonstrated for the first time a direct association of Crk-II with a member of the insulin receptor family [19]. Recent studies implied a temporal tightly regulated IGF-IR catalysed phosphorylation/dephosphorylation cascade of Crk-II as a requirement for terminal differentiation but not concomitant clonal expansion during the differentiation of NIH3T3-L1 preadipocytes into adipocytes [28].

To elucidate whether differences in the insulin and IGF-1 triggered signals might be based upon differential and variable capabilities of the respective receptors to directly associate with Crk isoforms we carried out investigations relying on the yeast two-hybrid method. Mutational analysis of Crk-II revealed a differential pattern of interaction of this adapter protein with the insulin receptor when compared to the IGF-I receptor and thus provides a first evidence for a direct interplay of Crk proteins and members of the insulin receptor family in a cellular environment.

Materials and methods

cDNA plasmid constructs. cDNAs from the neuroblastoma cell line SH-SY5Y and a neuroepithelioma cell line (SK-N-MC) were employed as sources for the adapter primer driven PCR amplification of full length human Crk-L and Crk-II, respectively. Using compatible ends a *Mfe*I/*Xho*I restriction fragment of Crk-L was cloned into the *Eco*RI/*Xho*I digested yeast expression vector pB42AD (pB42-Crk-L). Likewise, a PCR with a *Mfe*I forward adapter primer and a *Sal*I reverse adapter primer yielded two fragments of 969 and 799 bp, representing Crk-II and Crk-I, respectively. PCR products were digested with the indicated enzymes and ligated into the *Eco*RI/*Xho*I digested pB42AD (pB42-Crk-II and pB42-Crk-I). Sequence integrity was verified by DNA sequencing and alignment to the respective GenBank reference entries (Crk-II, NM_016823; Crk-I, NM_005206). In an initial attempt to amplify the Crk-L cDNA by PCR three independent nucleotide substitutions were introduced by a fluke within the sequence region encoding the Crk-LSH2 domain (A607G, T666C, and A723G; of NM_005207). The missense mutations are predicted to cause amino acid substitution His33Arg, Ser53Pro, and Lys72Arg. This construct was cloned as described for the wild type Crk-L cDNA and designated Crk-LSH2(Mut). Deletion mutant of the N-terminal Crk-IISH3 domain was generated by restriction endonuclease digest of pB42-Crk-II with *Bgl*II and *Bst*EII. The protruding 5′-ends were filled in using T4 DNA polymerase and the resulting plasmid was self-ligated [pB42-Crk-IIΔSH3(N)] followed by DNA sequencing to assure in-frame ligation. The resulting protein lacks amino acids Leu-151 to Gly-203 and contains an additional arginine residue at the site of DNA ligation. pB42-Crk-IIΔSH3(C) was created by PCR as the wild type construct but differing in the reverse primer using pB42-Crk-II as template. This primer combination amplified a fragment excluding the sequence encoding for the C-terminal SH3 domain but including the triplet coding for tyrosine 221. In addition, a new stop codon was introduced resulting in peptide chain termination after Pro-248. Likewise, an even more C-terminal truncated construct was generated in the same way as pB42-Crk-IIΔSH3(C) representing only the SH2 domain. A stop codon was introduced resulting in peptide chain termination after Leu-114 and the resulting plasmid was designated as pB42-Crk-IISH2. BTM-tpr-met-Gab1ΔPH and BTM-tpr-met-Gab1ΔPH-ΔCBR LexA fusion plasmids were kindly provided by Dr. U. Schaeper (Max Delbrück Center for Molecular Medicine, Berlin, Germany). These cDNAs were excised from their original vectors by *Pme*I and *Sal*I, and ligated into the same restriction sites within pLexA to adopt the fusion proteins to the two-hybrid system used in this study.

Receptor cDNAs were cloned into the yeast expression vector pLexA. The insulin receptor cDNA was kindly provided by Dr. E. van Obberghen (INSERM U145, Nice, France), that one for IGF-I receptor was a generous gift of Dr. R. Furlanetto (Medical University of South Carolina, SC). The IRR construct was cloned from the full

length cDNA described earlier [29]. All LexA-receptor fusions comprised the intracellular domains of the receptors (pLexA-IRR-C: amino acids 944–1297 of IRR, NP_055030; pLexA-InsR-C: amino acids 979–1382 of insulin receptor, NP_000199; and pLexA-IGFIR-C: amino acids 959–1367 of IGF-I receptor, NP_000866). The QuickChange XL-Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to introduce a two nucleotide substitution within the pLexA-InsR-C construct resulting in an amino acid exchange from lysine to alanine [K1057A, using another numbering scheme frequently referred to as K1030A; pLexA-InsR-C(KD), "kinase dead"]. All steps were performed according to Stratagene's protocol. Sequences encoding the most proximal cytoplasmic parts of the insulin receptor and the IGF-I receptor comprising a stretch of 13 and 12 amino acids, respectively, were mutually exchanged. pLexA-InsR-IGFIR(N), that contains the IGF-IR N-terminal amino acid stretch (His-959-Val-971), was created by PCR amplification using a forward primer consisting of a 5′-*Eco*RI-site, the IGFIR(N) sequence, and the adjacent insulin receptor sequence for template annealing. Reverse primer was complementary to a sequence within the insulin receptor downstream to a *Dra*III site. The resulting 1007 bp PCR fragment was digested with *Eco*RI and *Dra*III, and ligated into pLexA-InsR-C linearized by the same restriction enzymes. pLexA-IGFIR-InsR(N) comprising Leu-979-Pro-990 out of the insulin receptor was constructed as exemplified for the pLexA-InsR-IGFIR(N) plasmid. Primer sequences and more detailed cloning protocols are available upon request.

Yeast two-hybrid assays. Basically, all experiments were performed according to Clontech's protocol (Clontech BD Biosciences, Palo Alto, CA). Yeast strain EGY48 was transformed with plasmids using polyethylene glycol and lithium acetate. Transformants were grown on selective media containing glucose as the carbon source. To drive expression from the pB42-AD plasmid colonies were transferred to selective solid SD media where glucose has been replaced by galactose/raffinose. All yeast incubations were performed at 30 °C. In an initial transformation the p8op-lacZ reporter plasmid was introduced into EGY48 and maintained by uracil selection (EGY48/LacZ). β-Galactosidase assays were performed as follows. For colony-lift filter assays colonies were transferred directly onto filter paper placed on SD agar media with galactose/raffinose as carbon source. After overnight incubation filters were frozen in liquid nitrogen for 15 s and thereafter placed onto a filter presoaked with 2.5 ml Z-buffer/X-gal/β-mercaptoethanol (60 mM $Na_2HPO_4 \cdot 7H_2O$; 40 mM $NaH_2PO_4 \cdot H_2O$; 10 mM KCl; 1 mM $MgSO_4 \cdot 7H_2O$; pH 7.0; 0.27% (v/v) of 2-mercaptoethanol; and 0.03% (w/v) X-gal) followed by incubation at 30 °C. Colonies were checked periodically for blue colour formation. For documentation filters were dried using a Gel Dryer 543 (Bio-Rad Laboratories, Hercules, CA) and digitized using an office scanner. Induction of the EGY48 *LEU2* reporter gene was performed by streaking yeast transformants onto SD agar media lacking leucine and supplemented with galactose/raffinose as carbon source. Progression of colony growth was observed for 2–6 days.

Results and discussion

Crk proteins have been demonstrated to be phosphorylated in many cell types and growth factor stimulation elevates rapidly the level of Crk tyrosine phosphorylation. While changes of Crk phosphorylation after insulin or IGF-1 stimulation are well described, the way by which this is achieved remains in most instances in the dark. Crk-II has been shown to be a direct substrate of the IGF-I receptor in vitro [19]. In these experiments phosphorylation of GST-Crk-II fusion proteins was dependent on the Crk-IISH2 domain which has been proposed to confer a transient association with the receptor juxtamembrane region. On the other hand, insulin stimulated phosphorylation of Crk-II is concomitant with the de-repression of Raf-1 kinase resulting in activation of the MEK/ERK cascade in CHO cells [24]. The question how activation of the insulin receptor leads to Crk phosphorylation is not yet dissolved. Furthermore, Crk assembly with activated tyrosine kinase receptors may be mediated by docking molecules or by direct association. In order to study whether IGF-I receptor and insulin receptor exhibit the same capabilities to associate with Crk isoforms we have employed a yeast two-hybrid approach. Both receptors were used as truncated proteins consisting of the cytoplasmic part of the receptor β-subunits fused to the LexA DNA-binding domain. Since such constructs are constitutively active, they display docking sites for phosphotyrosine dependent interaction partners.

Association of insulin receptor and IGF-I receptor with Crk isoforms

Yeast strain EGY48/LacZ was co-transformed with pLexA-receptor constructs and pB42-Crk isoforms Crk-II, Crk-I or Crk-L. A summary of all constructs used in this study is given in Fig. 1. Activation of the β-galactosidase reporter could only be detected in strains expressing the wild type insulin receptor (Fig. 2). Blue colour formation in yeast strains co-expressing the IGF-IR was barely visible. These data indicate that the insulin receptor forms stable complexes with Crk-L leading to reporter gene activation in the yeast two-hybrid assay. As opposed to the insulin receptor IGF-I receptor fails to interact with Crk-L under these conditions. Similarly, the third member of the insulin receptor family, IRR, does not associate with Crk-L (Fig. 2, lower frame). This might be caused by a lack of constitutive autophosphorylation of the IRR cytoplasmic part in yeast[1]. To exclude this possibility interaction assays were performed with a constitutively activated IRR chimeric receptor containing the IGFIR tyrosine kinase domain. Again, no association with Crk was detectable (data not shown). Replacement of alanine for lysine at position 1057 (K1057A) in the insulin receptor, a residue that is probably involved in ATP binding or γ-phosphate transfer, leads to a non-functional tyrosine kinase [InsR-C(KD)]. As expected and shown in Fig. 2 the "kinase dead" mutation completely abolished the interaction with Crk-L. This result is in line with the observation that the direct interaction of Crk proteins with receptor tyrosine kinases requires receptor phosphorylation [15,30]. As a positive control

[1] Manuscript in preparation.

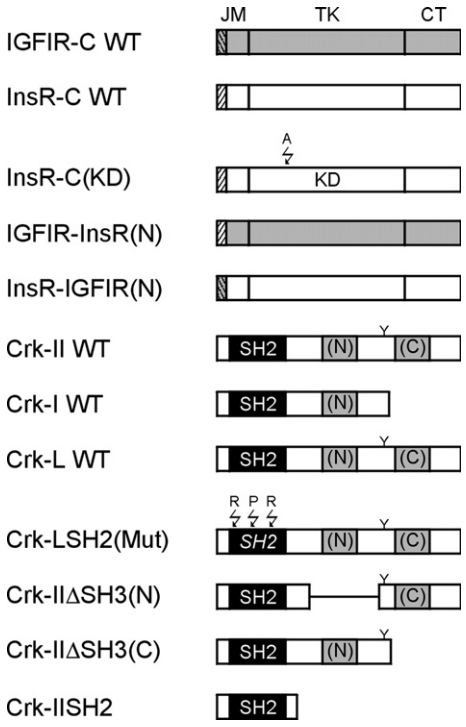

Fig. 1. Schematic overview on the constructs used in the yeast two-hybrid assays. The upper part represents the organization of the cytoplasmic portion of the wild type insulin receptor (InsR-C WT) and the IGF-I receptor (IGFIR-C WT). The illustrated domains are JM, juxtamembrane domain; TK, tyrosine kinase domain; and CT, C-terminal tail. Hatched boxes mark the most N-terminal parts of the receptor constructs and are displayed differently for insulin receptor and IGF-I receptor. Below the wild type receptors the "kinase dead" mutant of the InsR as well as the N-terminal exchange constructs are presented. In the lower part Crk wild type isoforms are schematically displayed. Crk-LSH2(Mut) represents a mutant characterized by a triple amino acid substitution within the SH2 domain followed by Crk-II deletion mutants. Black box, SH2 domain. Grey boxes, SH3 domains. (Y), Tyrosine residue. N-terminal LexA (receptors) and B42 peptide fusions (Crk) are not displayed.

for the integrity of the B42-Crk-L fusion protein the interaction of Crk-L with a constitutively phosphorylated Gab1 was tested (Fig. 2). Gab1 has been shown to associate with Crk-L in response to HGF/SF stimulation. Since Gab1 is a direct substrate of the c-Met (HGF/SF receptor) tyrosine kinase this ternary complex can be mimicked in its activated state in yeast by fusion of a constitutively activated Met receptor kinase (tpr-met) to Gab1 (tpr-met-Gab1) [31]. The association of this construct with Crk-L can be abrogated by deletion of a cluster of six Crk consensus-binding sites (the Crk-L binding region, CBR; Fig. 2, tpr-met-Gab1ΔCBR) [31]. The results obtained with Crk-L interaction induced β-galactosidase reporter activation were reproduced in assays in which the leucine auxotrophy of EGY48 was complemented by activation of the *LEU2* reporter (Fig. 3A). Although association of the IGF-I wild type receptor with Crk-II in a β-galactosidase assay was barely detectable (Fig. 3B, lowest row)

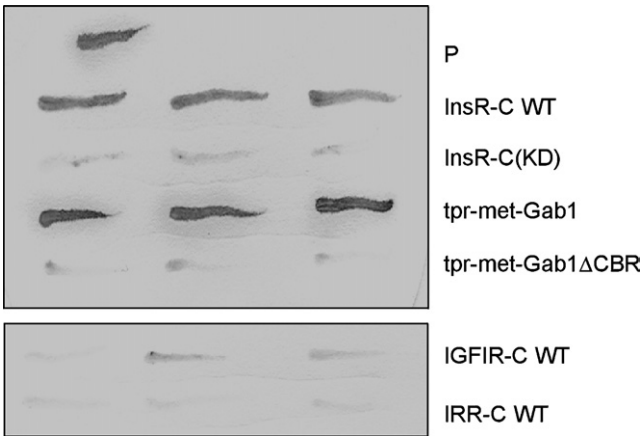

Fig. 2. Crk-L interacts with a functional insulin receptor, but not with IGF-I receptor in a yeast two-hybrid assay. β-Galactosidase filter assays were performed in triplicates as described in Materials and methods. Filters were dried and photographed after 4 h of incubation. Figure shows one of at least two independent experiments. (P) Assay positive control (pLexA-p53 with pB42-T); tpr-met-Gab1, positive control for Crk-L interaction; and tpr-met-Gab1ΔCBR, negative control.

IGFIR was capable of activating the leucine reporter after 5 days of incubation (Fig. 3A, sector 3). However, this interaction was visibly much less pronounced when compared to the insulin receptor (Fig. 3A, sector 2) and might be due to the higher sensitivity of this assay.

Lack of a N-terminal proline rich motif in the IGF-I receptor does not account for the failure in Crk recruitment

To evaluate a possible involvement of the CrkSH3 domains in the interaction with the insulin receptor a comparative sequence analysis for putative SH3-binding domains was performed. Filtered analysis using the "Eucaryotic Linear Motif" resource (ELM) server[2] predicted a type I SH3 recognition motif ranging from Lys-981 to Pro-987 (corresponding to residue −15 to −9 from the NPEY motif where $N = 0$) within the insulin receptor [32]. Since this motif is not conserved among the other two members of the insulin receptor family possible participation of this sequence in association with c-Crk was investigated. To this end the region of interest in the insulin receptor was replaced by the corresponding part out of the IGF-I receptor and vice versa. As shown in Fig. 3B interaction of the receptors with Crk-II was not affected by the domain exchange. Neither the IGFIR containing the putative InsR SH3 recognition sequence did elevate in its Crk interaction nor was the association of Crk-II with an insulin receptor lacking this sequence impaired by the replacement. Thus, the KRQPDGP motif juxtaposed upstream to the

[2] http://elm.eu.org/basicELM/

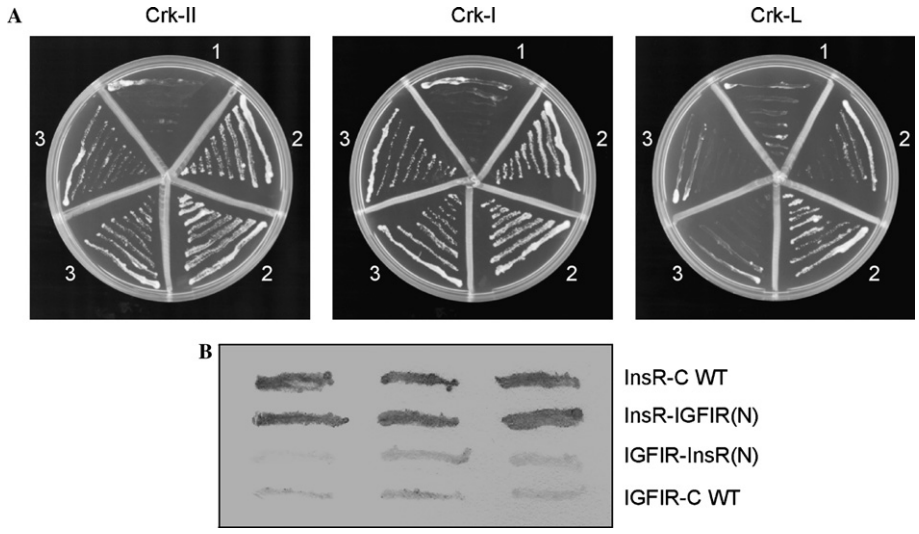

Fig. 3. (A) Differential interaction of insulin receptor and IGF-I receptor with Crk isoforms. Colonies were grown on leucine depleted medium in duplicates. Figure shows one of at least two independent experiments. Plates were photographed after 5 days of incubation at 30 °C; (1) InsR-C(KD), (2) InsR-C WT, and (3) IGFIR-C WT. (B) β-Galactosidase filter assay of Crk-II with WT receptors or receptor constructs with exchanged N-terminal sequences. Introduction of a putative SH3-binding site of the insulin receptor into the IGF-I receptor is without effect. β-Galactosidase filter assays were performed in triplicates as described in Materials and methods. Filters were dried and photographed after 5 h of incubation. Figure shows one of at least two independent experiments.

NPEY sequence in the insulin receptor is probably not a functional SH3 recognition site, at least for the CrkSH3 domains.

Interaction of the c-CrkSH2 domain with the IGF-I receptor is hindered by the C-terminal part of c-Crk

To further dissect the contribution of the functional domains of Crk-II and Crk-L to the association with insulin receptor and IGF-I receptor various deletion mutants were employed in the interaction assays. In addition, a Crk-L construct mutated in its SH2 domain was used (for construct specification see Fig. 1). As the experiments with the receptor "kinase dead" mutant InsR-C(KD) suggested association of InsR-C with Crk-L is conferred by a phosphotyrosine-SH2 domain interaction. This assumption is confirmed by using Crk-LSH2(Mut). Mutations within the SH2 domain of Crk-L abolish the interaction induced β-galactosidase reporter activation (Fig. 4A). Deletion of the SH3(N) or SH3(C) domains of Crk-II as well as a truncated protein consisting of only the SH2 domain were without visible effect on the interaction with the insulin receptor compared to the wild type receptor in the β-galactosidase assays. To assure a functional IGFIR-C, i.e., phosphorylated receptor, interaction with a B42-IRS1 fusion protein was tested (Fig. 4A, IGFIR-C WT pane, control). As expected IGF-I receptor associates strongly with IRS1. In contrast to the insulin receptor, association of the IGF-I receptor with Crk-II constructs lacking the SH3(C) domain was strikingly changed compared to wild type receptor. Whereas the association with Crk-IIΔSH3(N) was barely detectable similar to Crk-II WT β-galactosidase reporter activity was evident when using Crk-IISH2 (lacking Tyr-221) or Crk-IIΔSH3(C) (retaining Tyr-221). In addition, activation of the leucine reporter basically paralleled the results attained in the β-galactosidase filter assays (Fig. 4B). In these approaches enhancement of the interaction by deletion of the C-terminal SH3 domain was less pronounced when compared to wild type Crk-II. Instead, yeast strains expressing the Crk-IISH2 domain displayed the strongest interaction with the IGF-I receptor. These data demonstrate that (1) IGF-I receptor displays a docking site for the Crk-IISH2 domain, and (2) stable interaction of Crk-II with IGFIR-C is hindered by the presence of the C-terminal region comprising the C-terminal SH3 domain.

Crk-II phosphorylation by and association with the IGF-I receptor in vitro has been shown previously [19]. The interaction was reported to depend on the presence of the CrkSH2 domain which facilitates specific association with phosphotyrosine residues within the juxtamembrane region of the activated IGF-I receptor. Our data obtained in a cellular environment are in line with these in vitro results. Moreover, we provide evidence that both the insulin receptor and the IGF-I receptor display a docking site for Crk isoforms. Interaction between the receptor and Crk proteins depends on the intrinsic receptor tyrosine kinase and a functional CrkSH2 domain. Although not tested in the present study we propose the same phosphotyrosine residues to be involved in the interaction with the insulin receptor as has been described for the IGF-I receptor, namely

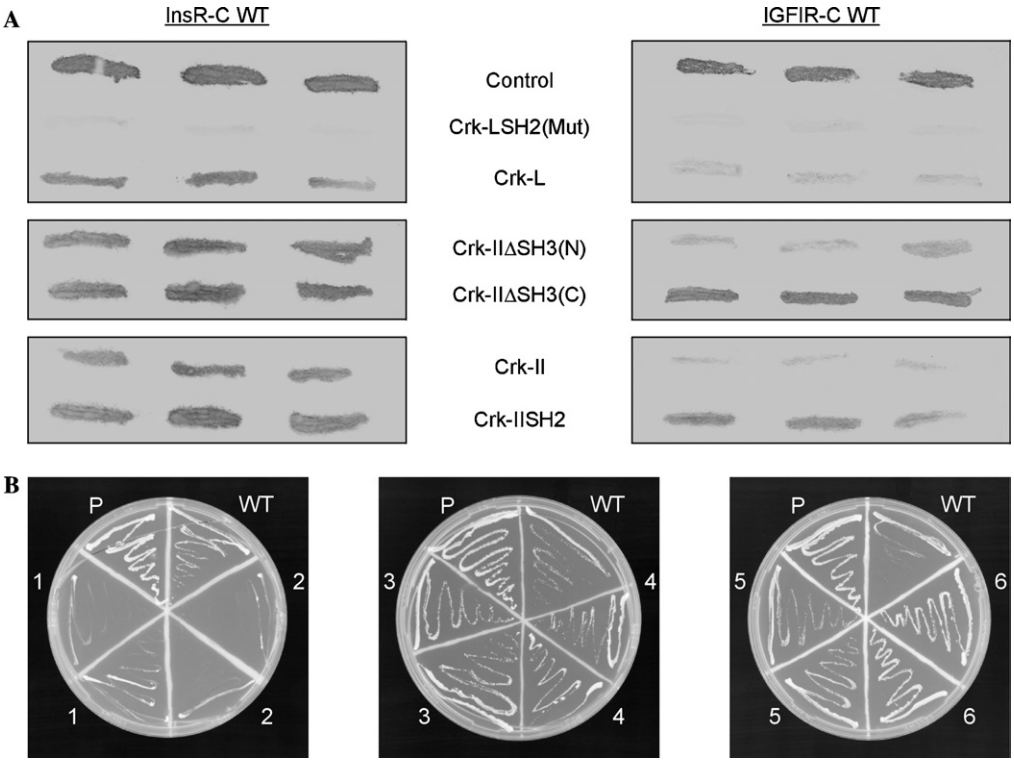

Fig. 4. Truncation of the C-terminal portion of Crk-II facilitates interaction with the IGF-I receptor. (A) β-Galactosidase filter assays were performed twice in triplicates. Filters were dried and photographed after 3 h of incubation. For explanation of the constructs used see the text and Fig. 1. Control in the left InsR-C WT pane is pLexA-tpr-met-Gab1 × pB42-Crk-L, control in the right IGFIR-C WT pane is pLexA-IGFIR-C × pB42-IRS1. (B) Growth of yeast strains co-transformed with the indicated plasmids on leucine depleted medium. Colonies were grown on leucine depleted medium in duplicates [except for controls P and WT]. Figure shows one of at least two independent experiments. Plates were photographed after 5 days of incubation at 30 °C; IGFIR-C WT co-transformants with (P) IRS-1, (WT) Crk-II WT, (1) Crk-LSH2(Mut), (2) Crk-L WT, (3) Crk-IIΔSH3(C), (4) Crk-IIΔSH3(N), (5) Crk-IIΔSH3(C), and (6) Crk-IISH2.

that of the NPEpY motif and eventually tyrosine at position −4 N-terminal from the NPEpY motif. This assumption appears to be reasonable because the NPEY motif is completely conserved and the surrounding sequence displays only minor differences.

Despite comparable capabilities of the insulin receptor and the IGF-I receptor to associate with the Crk-IISH2 domain the results obtained when using full length Crk-II or SH3 deletion mutants differed significantly. Most interestingly, insulin receptor associated stably with full length Crk proteins in the yeast two-hybrid experiments and this interaction was not compromised by any of the tested deletion mutants. In contrast, IGF-I receptor interaction with full length Crk-II was diminished or even below the detection limit of the β-galactosidase assays. Phosphorylated tyrosine-221 of Crk-II has been reported to serve as a docking site for the SH2 domain residing in the same protein [33]. This intramolecular fold has at least two consequences. First, effector molecules occupying the SH3(N) domain might be swamped out, and second, the SH2 domain is not longer available for interaction with other tyrosine phosphorylated proteins. Confirming this model phosphorylation induced dissociation of Crk-II from the activated IGF-I receptor has been proposed [19]. This conformational change might also be the reason for the difficulties to detect interaction between IGF-I receptor and Crk-II in the presented yeast two-hybrid studies. Surprisingly, deletion of the SH3(C) domain conferred association with the IGF-I receptor to about the same extent as the employment of the SH2 domain alone (Fig. 4). This suggests an inhibitory function for this region of Crk-II. Some reports of studies using deletion and point mutants of Crk appear to support such an idea [34,35]. Alternatively, deletion of the entire C-terminal sequence 28 amino acid residues downstream from the major phosphorylation site of Crk-II might obstruct the phosphotyrosine moiety for intramolecular interaction with the SH2 domain resulting in stabilized accessibility of this module for IGF-I receptor docking. It is of importance to point out that the interaction of the insulin receptor with Crk-II was not affected by the presence of the tyrosine 221 or the C-terminal SH3 domain. Mutational analysis of the proline rich sequence residing between the transmembrane region and the highly conserved LpYAS[S/V]NPEpY motif in the insulin receptor argues against a specific interaction stabilizing effect of the N-terminal adjacent sequence

(Fig. 3B). Other portions of the insulin receptor (e.g., the five amino acid insertion C-terminal of the NPEY motif) might contribute to the stabilization of InsR–Crk association. On the other hand, it is conceivable that Crk is a better substrate for the IGF-IR tyrosine kinase than for the insulin receptor. In this case the equilibrium of SH2 interactions would shift from receptor association toward the intramolecular interaction. It should be noted that both tyrosine residues within the juxtamembrane region implicated in SH2 recognition do not conform to the canonical CrkSH2 ligand sequence, which has been described as pYXXP [10]. Thus, we propose a model in which IGF-1 stimulated Crk phosphorylation tends to promote the intramolecular Crk-IISH2 occupation (with all consequences for SH3(N) mediated interactions) whereas insulin receptor activation supports a more stable receptor–Crk-II complex. Regardless of whether this effect is achieved by a higher affinity of the phosphorylated insulin receptor to the CrkSH2 domain or by the IGF-I receptor being a more effective kinase for the Crk substrate or both the outcome would be a sequestration of the adapter protein to the insulin receptor compartment compared to re-organization of IGF-1 induced Crk signalling complexes in a receptor dissociated state. Phosphorylation of Crk-II in response to insulin might in addition or preferentially be catalysed by other enzymes, probably cytoplasmic non-receptor tyrosine kinases, that are activated by the insulin stimulus. Under native conditions in mammalian cells the situation is further complicated by the presence of other adapter and docking molecules that compete with Crk-II for phosphotyrosine-binding sites. One should keep in mind that the proposed CrkSH2 recognition sequence within the insulin- and IGF-I receptor is a genuine PTB ligand motif which binds the respective docking proteins with much higher affinity than Crk does. Inversely, canonical CrkSH2-binding sites are found clustered in many proteins (i.e., p130Cas, paxillin) representing preferred docking sites for Crk proteins.

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