14-3-3 Proteins interact with the insulin-like growth factor receptor but not the insulin receptor[1]

Richard W. FURLANETTO*[2], Bhakta R. DEY†, Wiodzimierz LOPACZYNSKI† and S. Peter NISSLEY†

*Strong Children's Research Center, Department of Pediatrics, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, U.S.A., and †Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.

We have used a yeast two-hybrid system to identify proteins which bind to the cytosolic portion of the type 1 insulin-like growth factor (IGF) receptor (IGFIR) but not the insulin receptor (IR). This analysis identified 14-3-3β and ζ proteins. 14-3-3β also binds to the IGFIR but not the IR *in vitro* and 14-3-3–IGFIR complexes are present in insect cells overexpressing the IGFIR cytoplasmic domain. 14-3-3 proteins are substrates of the IGFIR in the yeast system and *in vitro*. The interaction of 14-3-3 with the IGFIR requires receptor-kinase activity and maps to the C-terminus of the receptor, but does not depend on tyrosine residues in this or the juxtamembrane regions. Instead, the binding maps to serine residue 1283 and requires phosphorylation of this residue. 14-3-3 proteins are phosphoserine-binding proteins which have been shown to interact directly with components of the mitogenic and apoptotic signalling pathways, suggesting that they participate in growth regulation. Our findings suggest that 14-3-3 proteins may play a role in IGFIR signal transduction and may contribute to the differences in IGF and IR signalling capabilities.

INTRODUCTION

The insulin-like growth factors, IGF-I and IGF-II, play an important role in growth and development (reviewed in [1]). These effects of the IGFs are mediated through the type 1 IGF receptor (IGFIR) (reviewed in [2]). This receptor is a member of the tyrosine kinase family of growth-factor receptors and is closely related to the insulin receptor (IR). Binding of the cognate ligands activates these receptors and results in their autophosphorylation. This creates motifs containing phosphotyrosine residues which are recognized by cytosolic proteins involved in signal transduction by these receptors (reviewed in [3]). Some of these proteins, such as IRS-1 [4], IRS-2 [5] and Shc [6], are also substrates of these receptors. It is noteworthy that the IGFIR and IR interact with similar cytosolic proteins, suggesting that they share a common signal-transduction pathway. Functionally, however, the IGFIR is more effective than the IR in stimulating mitogenesis [7]. This difference in signalling potential maps to the C-termini of these receptors and suggests that these regions interact with different cytosolic effector molecules [8–13]. However, no protein which interacts selectively with the IGFIR has been reported.

Understanding of the mechanism by which the IGFs elicit their mitogenic effect and how this differs from insulin signal transduction requires the identification and characterization of the proteins which interact with the IGFIR *in vivo*. To identify such proteins we have used a yeast two-hybrid assay, the interaction trap [14], to screen a human fetal brain cDNA library for proteins which bind selectively to the cytoplasmic domain of the IGFIR. The analysis identified two members of the 14-3-3 family of proteins. 14-3-3 proteins are phosphoserine (pSer)-binding proteins which have been shown to interact directly with products of oncogenes and components of the mitogenic and apoptotic signalling pathways, suggesting that 14-3-3 proteins may play a role in growth regulation [15–24].

EXPERIMENTAL

Materials

The yeast strains (EGY48 and EGY188) and expression plasmids used for the interaction trap were obtained from Dr. Roger Brent [14]. The human fetal brain activation domain (AD) fusion library used in these studies was obtained form Dr. R. Brent and Dr. D. Krainc (Massachusetts General Hospital, Boston, MA, U.S.A.). NIH-3T3 cells overexpressing the IGFIR (NWTc48 cells) [9] or the IR (3006 cells) [25] were obtained from Dr. Derek LeRoith and Dr. Simeon Taylor (National Institutes of Health, Bethesda, MD, U.S.A.) respectively. The rabbit antiserum to the C-terminal region of the IGF-1 receptor (αIGFIR) was produced as described previously [26]. A rabbit antiserum to the C-terminal region of the insulin receptor (αIR) was a gift of Dr. J. N. Livingston (Bayer Corporation, West Haven, CT, U.S.A.). Other reagents were obtained from commercial sources as indicated in the text and Figure legends. Phosphorylated and non-phosphorylated peptides corresponding to residues 1269–1289 of the IGFIR were purchased from Dr. M. Berne (Tufts University, Boston, MA, U.S.A.); the peptides were purified by HPLC and their identities confirmed by MS.

Yeast two-hybrid plasmid construction

The construction of the kinase-active (i.e. wild-type) LexA–IGFIR cytoplasmic domain bait hybrid [LexA–IGFIR(WT)] and the kinase-negative mutant bait hybrid in which Lys-1003 in the ATP-binding domain of the IGFIR is changed to Arg

Abbreviations used: AD, activation domain; DTT, dithiothreitol; ECL, enhanced chemiluminescence; IGF, insulin-like growth factor; IGFIR, type 1 IGF receptor; IR, insulin receptor; IRS, insulin receptor substrate; GST, glutathione *S*-transferase; LexA–IGFIR(WT), kinase-active IGFIR cytoplasmic domain bait hybrid; LexA–IGFIR(KR), kinase-negative mutant bait hybrid in which Lys-1003 in the ATP-binding domain of the IGFIR is changed to Arg; pSer, phosphoserine; Raf, c-raf-1.

[1] This work was presented in part at the Tenth International Congress of Endocrinology, San Francisco, CA, U.S.A., June 12–15 1996 (Abstract OR31-6).

[2] To whom correspondence should be addressed.

[LexA–IGFIR(KR)] has been described previously [26]. The wild-type LexA–IR bait hybrid containing IR residues 941–1343 was constructed using a similar approach. A chimeric LexA–IGFIR–IR bait hybrid plasmid in which residues 936–1216 of the IGFIR are linked to residues 1231–1343 of the IR was constructed by the method of Tartare et al. [27]. This approach utilizes a unique *Fsp*I site in a conserved region of the kinase domains of the two receptors. Mutant LexA–IGFIR bait hybrid plasmids were generated by PCR using appropriately altered primers. The AD–14-3-3β construct lacking a linker sequence was constructed by amplifying amino acid residues 1–246 of 14-3-3β by PCR and inserting this into the *Eco*RI and *Xho*I sites in pJG4-5 [14]. The sequences of all PCR-generated fragments were confirmed by the manual dideoxy or automatic ABI prism DNA sequencing.

Expression and purification of recombinant proteins

$(His)_6$-tagged IGFIR cytoplasmic domain

The region of the IGFIR encoding amino acid residues 936–1337 was amplified by PCR and the product was inserted into the *Bam*HI and *Pst*I sites of pBlueBacHis B transfer vector (Invitrogen). Sf9 cells were co-transfected with this construct and wild-type AcMNPV DNA (Invitrogen) and the recombinant virus was identified and purified. Sf9 cells were infected with recombinant virus at a multiplicity of infection of 10. After 72 h, cells were lysed and the $(His)_6$-tagged IGFIR cytoplasmic domain fusion protein was purified by chromatography on metal-affinity resin (Clontech or Qiagen) and eluted with 100–300 mM imidazole buffer, pH 8.0.

Glutathione *S*-transferase (GST)

pGEX-14-3-3β was constructed by cloning the insert in pJG-14-3-3β into the *Eco*RI and *Xho*I sites of pGEX-4T-1 (Pharmacia). The fusion protein was expressed in DH5α and purified according to the manufacturer's recommendations.

Yeast two-hybrid library screen

The human fetal brain cDNA library screen was performed using the two-step procedure recommended by Gyuris and Brent [14], as described previously [26]. In the first step, 0.5 μg of the library was used to transform yeast containing the LexA–IGFIR(WT) hybrid plasmid and transformants were selected for growth on medium lacking tryptophan (the pJG library plasmid has a Trp1 selectable marker) and containing glucose as the carbon source. In the second step, interactors were selected by plating approximately 10^7 primary transformants on to medium containing galactose (galactose induces expression of the AD-hybrid protein) and 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside but lacking leucine. After 4 days, approx. 100 of the fastest-growing and most intensely blue colonies were isolated and subcultured; this yielded about 80 pure clones with a galactose-dependent $LEU2^+/lacZ^+$ phenotype. These clones were sorted by PCR and restriction digestion. This analysis identified eight distinct cDNA inserts. The corresponding plasmids were isolated and tested for their ability to activate reporter-gene expression when co-expressed in yeast containing the LexA–IGFIR(WT), LexA–IGFIR(KR) or LexA–IR(WT) baits. Three of these clones activated both the lacZ and LEU2 reporters when co-expressed with the wild-type IGFIR cytoplasmic domain but not when co-expressed with the kinase-negative IGFIR mutant or the wild-type IR. These clones were found to encode 14-3-3β (two clones differing in the length of their 5′ untranslated regions) and 14-3-3ζ.

Binding studies *in vitro*

For binding studies *in vitro*, NIH-3T3 cells overexpressing the IGFIR (NWTc48) or IR (3006 cells) were employed. Subconfluent (60–80 %) monolayers were washed with PBS and incubated in serum-free Dulbecco's modified Eagle's medium for 16–24 h. The cells were then washed with warm PBS and Dulbecco's modified Eagle's medium containing 0.05 M Hepes (pH 7.5), 0.1 % (w/v) BSA and the appropriate hormone was added (10 nM IGF-I or 17 nM insulin). At various times after addition the cells were washed with cold PBS, lysed in buffer [20 mM Tris/HCl (pH 7.5)/137 mM NaCl/1 % (v/v) Nonidet P40/10 % (v/v) glycerol/1 mM $MgCl_2$/1 mM $CaCl_2$/1 mM PMSF] containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM Na_3VO_4 and 100 mM NaF, and the lysates were cleared by centrifugation at 16000 ***g*** for 30 min. Aliquots (100–200 μl) were incubated with glutathione–Sepharose containing either GST alone (5 μg) or GST–14-3-3β (10 μg) overnight at 4 °C. The beads were then washed with lysis buffer, boiled in SDS/PAGE sample buffer containing 100 mM dithiothreitol (DTT) and resolved by SDS/PAGE. The proteins were transferred to nitrocellulose (Immobilon-P, Millipore) and probed with an antibody specific for the C-terminus of the human IGFIR (αIGFIRβ) [26] or to phosphotyrosine (αPY) and appropriate horseradish peroxidase (HRP)-linked second antibodies. Detection was with enhanced chemiluminescence (ECL) (Amersham). The competitive binding experiments testing the phosphorylated and non-phosphorylated IGFIR peptides were performed similarly, except that 200 μM of the appropriate peptide was included in the incubation medium and the quantity of GST–14-3-3β was decreased to 2.5 μg per incubation.

RESULTS

Screening for proteins that interact selectively with the IGFIR

To identify proteins that interact selectively with the IGFIR, a yeast two-hybrid system, the interaction trap, was used to screen a human fetal brain cDNA library. The interaction trap employs a plasmid encoding a hybrid protein containing the LexA DNA-binding domain fused to the protein of interest (termed the bait hybrid) and a plasmid encoding a hybrid protein containing the B42 AD fused to the cDNA library. In this system an interaction between proteins is detected as galactose-dependent activation of the reporter genes *LEU2* and *lacZ*. This analysis identified three AD hybrid clones which activated the reporter genes when co-expressed with the LexA-bait hybrid containing the wild-type IGFIR cytoplasmic domain but not when co-expressed with the kinase-negative (i.e. KR) IGFIR bait hybrid or a bait hybrid containing the wild-type IR cytoplasmic domain. One of these clones encoded a fusion protein containing the entire coding sequence of the ζ isoform of 14-3-3 coupled to the activation domain by a 38-amino-acid linker peptide derived from the (normally) 5′ untranslated region of the cDNA. The other two clones encoded 14-3-3β; they differed in the length of the (normally) 5′ untranslated region of the cDNA and encoded linker peptides of 46 and 23 amino acids. These linker peptides differed from that encoded by the 14-3-3ζ construct, suggesting that the binding was due to the 14-3-3 proteins and not the linker peptides. This was confirmed using a 14-3-3β hybrid construct from which the entire linker sequence was deleted.

14-3-3 proteins bind to the IGFIR but not the IR *in vitro*

To determine if the IGFIR interacts with 14-3-3 *in vitro*, *in vitro* binding studies were performed. The results of two experiments

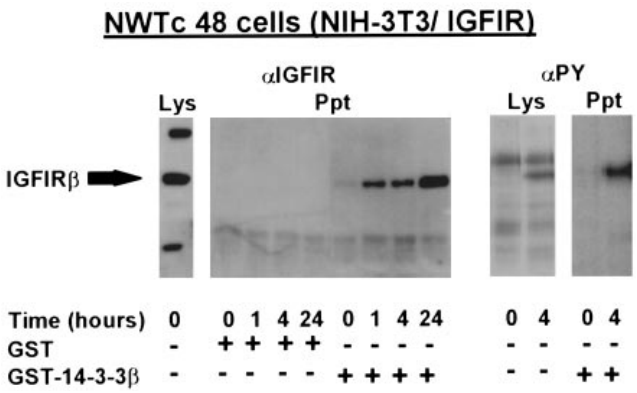

Figure 1 IGFIR binds to 14-3-3β *in vitro*

NIH-3T3 cells overexpressing the IGFIR (NWTc48 cells) were stimulated with 10 nM IGF-I and lysates were prepared at the times indicated. Aliquots were incubated with glutathione–Sepharose containing either GST alone (5 μg) or GST–14-3-3β (10 μg) overnight at 4 °C and the Sepharose was washed. Aliquots of the lysates (Lys) or Sepharose (Ppt) were boiled in SDS/PAGE sample buffer containing 100 mM DTT, resolved by SDS/PAGE and immunoblotted for either the IGFIR (αIGFIR) or phosphotyrosine (αPY). Detection was by ECL (Amersham). The position of the β subunit of the IGFIR (IGFIRβ) is indicated with an arrow.

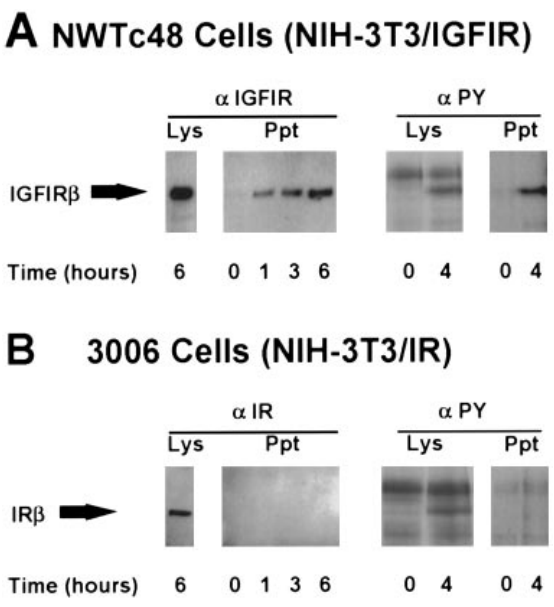

Figure 2 14-3-3β binds to the IGFIR but not the IR *in vitro*

NIH-3T3 cells overexpressing either (**A**) the IGFIR (NWTc48 cells) or (**B**) the IR (3006 cells) were stimulated with 10 nM IGF-I or 17 nM insulin respectively. Lysates were prepared at the indicated times. Aliquots were incubated with glutathione–Sepharose containing GST–14-3-3β (10 μg) and the Sepharose was washed. Aliquots of the lysates (Lys) or Sepharose (Ppt) were boiled in SDS/PAGE sample buffer containing 100 mM DTT, resolved by SDS/PAGE and immunoblotted for the IGFIR (αIGFIR), the IR (αIR) or phosphotyrosine (αPY). Detection was by ECL. The position of the β subunits of the IGFIR (IGFIRβ) and IR (IRβ) are indicated with arrows. The αPY analyses of the two cell lines were performed in parallel and are from the same immunoblots.

are shown in Figure 1 and Figure 2(A). Lysates prepared from IGF-I stimulated cells bound to the resin containing GST–14-3-3β, while lysates from quiescent cells had no binding activity. No binding was observed with resin containing GST alone. Binding

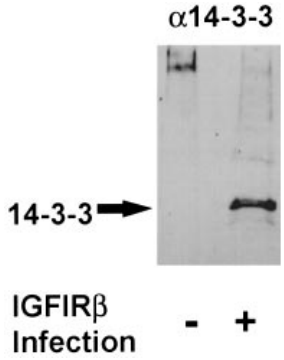

Figure 3 14-3-3 binds to the IGFIR cytoplasmic domain in Sf9 insect cells

Extracts prepared from 4×10^6 uninfected Sf9 cells or Sf9 cells expressing $(His)_6$-tagged IGFIR cytoplasmic domain were purified by metal-affinity chromatography. Aliquots (containing approx. 10 μg $(His)_6$-IGFIR) were boiled in SDS/PAGE sample buffer containing 100 mM DTT, resolved on a 5–20% gradient SDS/polyacrylamide gel and immunoblotted for 14-3-3 proteins. Detection was by ECL. The position of 14-3-3, determined using recombinant 14-3-3ζ, is shown with an arrow.

was detectable within 5 min of the addition of IGF-I and increased throughout the incubation, reaching maximum levels at 16–24 h (Figure 1). Thus the IGFIR binds to 14-3-3 proteins *in vitro* and this binding requires receptor activation.

The binding assay *in vitro* was also performed with the baculovirus-expressed IGFIR cytoplasmic domain. The baculovirus-expressed protein contains an N-terminal $(His)_6$-tag which allows purification by metal-chelate chromatography; it is partially phosphorylated when isolated from the insect cells. Similar to the mammalian receptor, the insect-cell-expressed IGFIR cytoplasmic domain bound to GST–14-3-3β but not to GST alone (results not shown).

To investigate the selectivity of the interaction *in vitro*, the binding studies were repeated using NIH-3T3 cells overexpressing the IR (3006 cells) (Figure 2B). As a positive control, NWTc48 cells were included in these experiments (Figure 2A). Immunoblot analysis of the whole-cell lysates showed that the cognate hormones stimulated approximately equivalent incorporation of phosphotyrosine into the β-subunits of the two receptors, indicating that the lysates from the two cell lines contained comparable amounts of activated receptors. However, no binding to the GST–14-3-3β resin was detected with lysates prepared from the 3006 cells using antisera to either the IR or to phosphotyrosine (Figure 2B). These results support a selective interaction of 14-3-3β with the IGFIR.

14-3-3 proteins bind to the IGFIR *in vivo*

To determine if the IGFIR also associates with 14-3-3 proteins *in vivo*, we examined the insect-cell-expressed receptor. When Raf is expressed in insect cells, it associates with endogenous 14-3-3 proteins in a kinase-dependent manner [20–22]. Extracts prepared from uninfected Sf9 cells and Sf9 cells expressing the $(His)_6$-tagged IGFIR cytoplasmic-domain fusion protein were purified by metal-affinity chromatography, subjected to SDS/PAGE and immunoblotting for 14-3-3 proteins. Purified extracts from receptor-expressing cells, but not uninfected cells, contained 14-3-3 (Figure 3). Thus 14-3-3 proteins also associate with the IGFIR in insect cells.

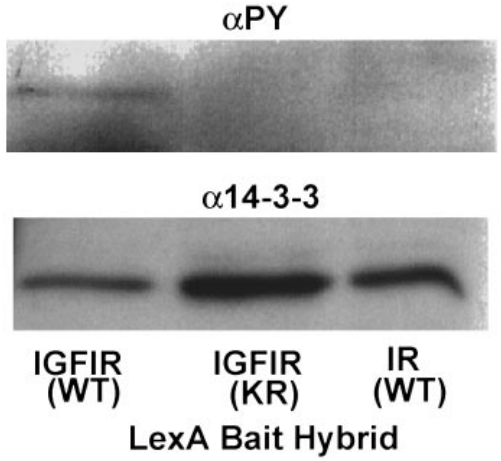

Figure 4 14-3-3β is phosphorylated by the IGFIR in yeast

Extracts were prepared from yeast co-expressing the AD–14-3-3β hybrid and either the IGFIR(WT) bait, the IGFIR(KR) bait or the wild-type IR bait by boiling for 10 min in SDS/PAGE sample buffer containing 100 mM DTT/1 mM PMSF/1 mM $NaVO_4$/100 mM NaF. Aliquots were resolved by SDS/PAGE (12% gels) and immunoblotted for either phosphotyrosine (αPY) or 14-3-3β (α14-3-3). Detection was by ECL.

14-3-3 proteins are substrates of the IGFIR in yeast and *in vitro*

Mammalian 14-3-3 proteins contain multiple tyrosine residues (nine of which are conserved among isoforms), and the τ isoform (Bap-1) has been shown to be phosphorylated on tyrosine residues by Bcr-Abl [16]. We have shown that Shc is phosphorylated by the IGFIR in the yeast interaction trap [26]. To determine if 14-3-3 proteins are also substrates of the IGFIR in yeast, extracts were prepared from yeast co-expressing the AD–14-3-3β hybrid and the wild-type IGFIR, the kinase-negative mutant receptor or the wild-type IR bait hybrids, and these were examined by immunoblotting for phosphotyrosine. Yeast expressing the wild-type IGFIR hybrid contained a phosphotyrosine-containing band corresponding to the AD–14-3-3β hybrid protein, whereas this band was absent in yeast expressing either the IGFIR(KR) mutant or the wild-type IR hybrid proteins (Figure 4). Thus, in yeast, the 14-3-3β hybrid undergoes IGFIR-directed tyrosine phosphorylation, which does not occur with the IR.

To determine if 14-3-3 proteins are also substrates of the IGFIR *in vitro*, lysates prepared from IGF-I-stimulated NWTc48 cells were incubated with GST–14-3-3β-Sepharose. After washing, the beads were incubated in the absence or presence of ATP and then examined by immunoblotting for phosphotyrosine. As shown in Figure 5(A), under these conditions GST–14-3-3β was tyrosine-phosphorylated. Similarly, when the purified baculovirus-expressed IGFIR cytoplasmic domain was incubated with ATP and recombinant 14-3-3ζ, the 14-3-3ζ protein was tyrosine-phosphorylated (Figure 5B). These findings indicate that 14-3-3 proteins are substrates of the IGFIR *in vitro*.

Mapping the 14-3-3 binding site on the IGFIR

We have shown previously that the yeast interaction trap can be used to map the sites of interaction of the IGFIR with IRS-1 and Shc [26]. To map the region of the IGFIR which interacts with 14-3-3, a series of mutant LexA–IGFIR bait hybrids was generated. The structures of the most informative constructs are shown in Figure 6 (left lower panel). These receptor baits were co-expressed with the AD–14-3-3β hybrid and the transformants

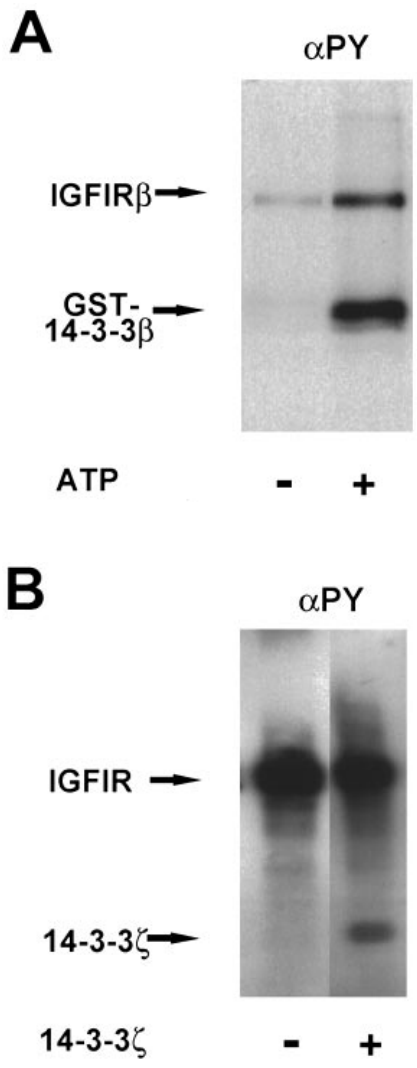

Figure 5 14-3-3 is phosphorylated by the IGFIR *in vitro*

(**A**) Lysates from IGF-I treated NWTc48 cells were incubated with glutathione–Sepharose containing GST–14-3-3β, washed and incubated for 60 min at 4 °C in the absence or presence of 0.1 mM ATP. (**B**) Purified baculovirus-expressed IGFIR cytoplasmic domain (50 ng) was incubated with 0.1 mM ATP in the absence or presence of recombinant 14-3-3ζ (1.5 μg) for 60 min at 4 °C. In both experiments the reactions were terminated by the addition of SDS/PAGE sample buffer containing 100 mM DTT and the samples were resolved by SDS/PAGE and immunoblotted for phosphotyrosine (αPY) (4G10, UBI). The positions of the IGFIR β-subunit (IGFIRβ), $(His)_6$-IGFIR (IGFIR), GST–14-3-3β and 14-3-3ζ (indicated with arrows) were determined by probing parallel blots with the respective antisera.

were assayed for reporter gene expression. The results, using the quantitative solution assay for lacZ expression, are shown in Figure 6 (right lower panel). As described earlier, co-expression of AD–14-3-3β with the wild-type LexA–IGFIR hybrid resulted in strong galactose-dependent activation of the LacZ reporter gene, while its co-expression with either the kinase-inactive mutant [i.e. IGFIR(KR)] or the wild-type IR did not activate the reporter. Mutation of Tyr-950 to phenylalanine (i.e. Phe-1337) had little effect on the activation of the reporter; this tyrosine is essential for the interaction of the receptor with IRS-1 and Shc [26]. However, deletion of the 27 C-terminal residues of the receptor (i.e. 1310-Tyr) decreased reporter expression by about 20% and deletion of the 93 C-terminal residues (i.e. 1244-Tyr) or

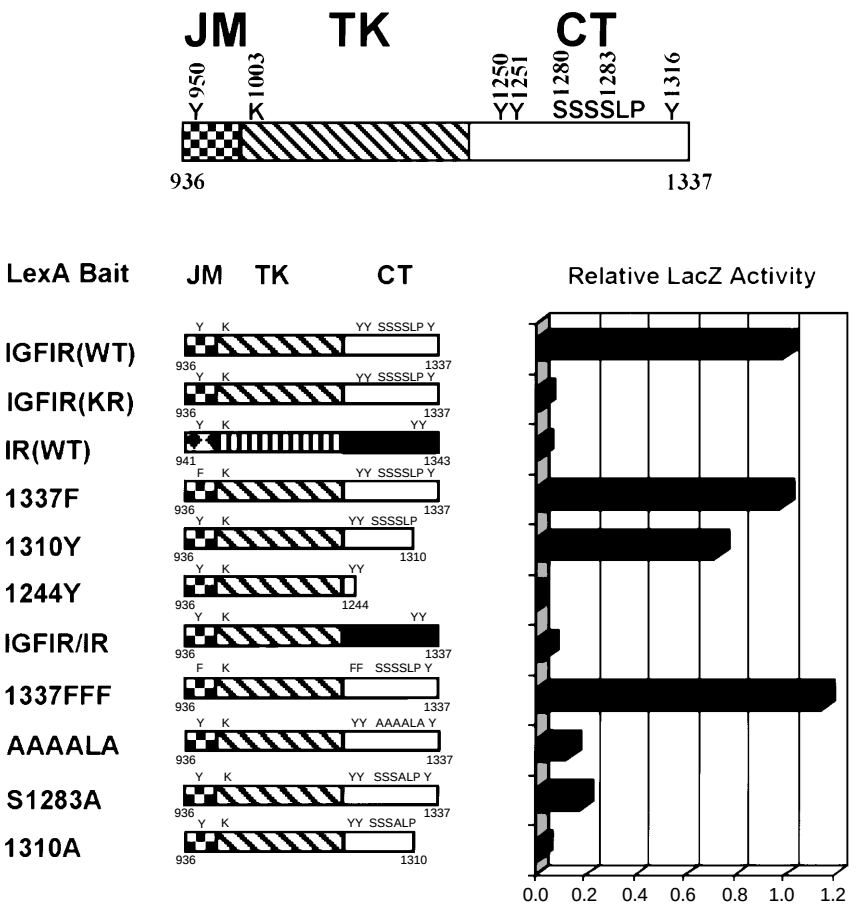

Figure 6 Mapping the IGFIR binding site for 14-3-3β in the yeast interaction trap

The upper panel shows a schematic representation of the IGFIR cytoplasmic domain and the locations of amino acid residues which were mutated in this study. The left lower panel shows schematic representations of the wild-type IGFIR cytoplasmic domain and informative mutatants. In the right lower panel is shown the lacZ activity of the constructs when co-expressed with the AD–14-3-3β plasmid. LacZ activity was measured using a quantitative solution assay [26], and activity is expressed relative to that of the wild-type IGFIR construct. Four clones from each transfection were assayed and the mean values are shown. JM, juxtamembrane region; TK, tyrosine-kinase domain; CT, C-terminal region.

replacement of the IGFIR C-terminus with that of the IR (i.e. IGFIR–IR) reduced LacZ expression to negligible levels.

The region of the IGFIR encompassing residues 1244–1310 is the most divergent region in the cytoplasmic domains of the IGFIR and IR (43% identical) but is highly conserved among species. This region of the IGFIR contains two tyrosine residues (Tyr-1250, Tyr-1251), which are not present in the IR and which have been implicated in IGFIR function [8,28]. To determine if these residues are important for interaction with 14-3-3 proteins, constructs in which one or both of these residues were changed to phenylalanine were generated and tested. Mutations of either or both of these tyrosines (i.e. 1337-Phe-Phe-Phe) had essentially no effect on reporter expression, indicating that these tyrosines are not necessary for this interaction.

The region of the IGFIR between residues 1244 and 1310 also contains a Ser-Ser-Ser-Ser-Leu-Pro sequence which is absent in the IR and which is similar to the consensus pSer-containing motif (Arg-Ser-Xaa-pSer-Xaa-Pro) present in other proteins which bind to 14-3-3. To determine if this motif is involved in binding to the IGFIR, constructs in which all four serines and the proline were changed to alanine (i.e. Ala-Ala-Ala-Leu-Ala), and in which only Ser-1283 was changed to alanine (i.e. Ser-1283 $\rightarrow$ Ala) were generated and tested. Each mutation resulted in an 80% to 90% decrease in lacZ reporter expression. The simultaneous mutation of Ser-1283 to alanine and deletion of the C-terminal 27 residues of the IGFIR (i.e. 1310-Ala) reduced reporter expression to negligible levels. These results indicate that Ser-1283 is a major determinant for the binding of 14-3-3 proteins to the IGFIR but that other residues, possibly located in the extreme C-terminus, may also contribute to the binding.

Phosphorylation of Ser-1283 is essential for binding of the IGFIR to 14-3-3 proteins

The data presented above indicate that Ser-1283 in the IGFIR is an important determinant for 14-3-3 binding. To determine if phosphorylation of this (or other) serine residue(s) is essential, competitive binding experiments were performed. In these experiments, phosphorylated and non-phosphorylated peptides, corresponding to residues 1269–1289 of the IGFIR, were tested for their ability to compete with the receptor for binding to 14-3-3β in the binding assay *in vitro*. This region of the receptor contains two Ser-Xaa-Pro motifs, which are potential binding sites, and peptides in which one or both of these serines were phosphorylated were tested. The results of a representative experiment are shown in Figure 7. Neither the non-phosphorylated peptide (P1) nor the peptide phosphorylated only at position 1272 (P3) competed with the IGFIR for binding. In contrast, the peptide

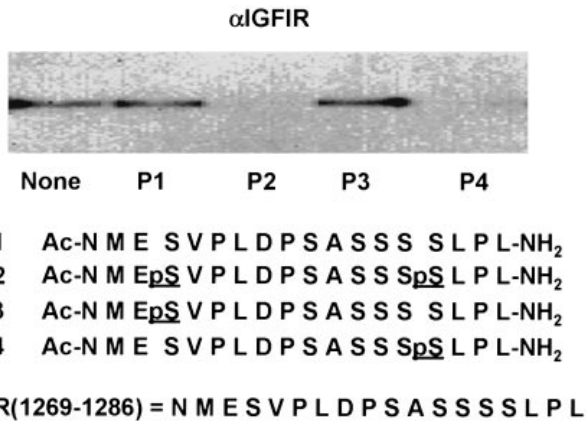

Figure 7 Phosphorylation of Ser-1283 is essential for binding of the IGFIR to 14-3-3β

NWTc48 cells were stimulated with IGF-I for 16 h and lysates were prepared. Aliquots were mixed with buffer only or the appropriate peptide (200 μM final concentration), incubated with glutathione–Sepharose containing GST–14-3-3β (2.5 μg) and the Sepharose was washed. Aliquots of the Sepharose were boiled in SDS/PAGE sample buffer containing 100 mM DTT, resolved by SDS/PAGE and immunoblotted for the IGFIR (αIGFIR). Detection was by ECL. The region of the gel containing the β subunit of the IGFIR is shown.

phosphorylated on both Ser-1272 and Ser-1283 (P2) and the peptide phosphorylated only on Ser-1283 (P4) inhibited binding. These data suggest that phosphorylation of Ser-1283 is essential for binding of the IGFIR to 14-3-3β.

DISCUSSION

These studies were undertaken to identify proteins that interact selectively with the IGFIR. To this end we used the yeast interaction trap to screen a human cDNA library. This analysis identified 14-3-3β and 14-3-3ζ. We have also shown that 14-3-3β binds to the IGFIR but not the IR *in vitro*. This observation indicates that the interaction observed in yeast is not an artifact resulting from the expression of normally cytosolic proteins in the nucleus or their expression as LexA and AD fusion proteins.

14-3-3 proteins are a highly conserved family of acidic proteins which are found in a wide range of organisms, including plants, yeast and mammals (reviewed in [15]). Seven mammalian isoforms have been identified (β, γ, ϵ, ζ, η, σ and τ). Some isoforms are expressed in cell-type or tissue-specific fashion (i.e. τ in T-cells and σ in keratinocytes) and most cells contain more than one isoform. All isoforms have molecular masses of about 30 kDa (about 250 amino acids) but these proteins exist as homo- and hetero-dimers *in vivo* [29]. Sequence analysis indicates that 14-3-3 proteins contain a mid-molecule annexin-like domain [30]. The crystal structure indicates a dimeric structure consisting of bundles of α-helices arranged around an amphipathic groove. Invariant residues line the groove, suggesting that it might form part of a ligand-binding surface [30,31].

Multiple activities have been reported for 14-3-3 proteins, including the activation of enzymes involved in neurotransmitter synthesis and the regulation of protein kinase C activity [15]. 14-3-3 proteins have been shown to interact directly with components of the mitogenic and aptoptic signalling pathways, including bcr and bcr-abl [16], cdc25 [17], Raf [18–22], BAD [23] and A20 [24]. They have also been shown to bind to a pSer-containing motif in Raf, and a similar motif (Arg-Ser-Xaa-pSer-Xaa-Pro) is present in many other proteins with which 14-3-3 associates [32]. These and other observations have led to the hypothesis that 14-3-3 proteins function both as adaptor molecules involved in the assembly of signalling complexes and as chaperones to protect other proteins from inactivation or to sequester them in the cytoplasm [23,24,29,31–34].

The interaction of 14-3-3 with the IGFIR requires receptor kinase activity both in yeast and *in vitro*, suggesting that receptor autophosphorylation is important. However, we found that Tyr-950, Tyr-1250, Tyr-1251 and Tyr-1316 are not necessary for the interaction in the yeast two-hybrid system. Instead, the interaction maps to Ser-1283 in the C-terminus of the receptor, and competitive binding experiments suggest that phosphorylation of this residue is essential for the interaction. Ser-1283 forms part of an Ser-Ser-Ser-Ser-Leu-Pro sequence which is similar to the consensus pSer-containing motif (Arg-Ser-Xaa-pSer-Xaa-Pro) identified in Raf, and in most other proteins which bind to 14-3-3 [23,24,32–34]. This suggests that serine phosphorylation also plays a role in the binding of 14-3-3 to the IGFIR. The IGFIR has been reported to undergo serine phosphorylation *in vivo*, but the biological effects of this modification are unknown [35]. Our observation that 14-3-3 proteins interact with the IGFIR in yeast indicates that either yeast expresses a serine kinase capable of phosphorylating the IGFIR or the phosphorylation is catalysed by the receptor itself.

14-3-3β and 14-3-3ζ are substrates of the IGFIR tyrosine kinase both *in vitro* and in the yeast two-hybrid system. Some 14-3-3 isoforms are phosphorylated on serine *in vivo* and the τ isoform has been shown to be phosphorylated on tyrosine by Bcr-Abl *in vitro* and *in vivo* [16]. It is noteworthy that 14-3-3ζ also binds to Bcr-Abl, but it is a poorer substrate, suggesting that there may be kinase-isoform specificity. We have not yet determined if the IGFIR also shows isoform specificity or which, if any, 14-3-3 isoforms undergo tyrosine phosphorylation in response to IGF stimulation *in vivo*.

We found that 14-3-3 associates with the IGFIR in insect cells overexpressing the receptor cytoplasmic domain. However, we have been unable to detect 14-3-3 in IGFIR immunoprecipitates of NWTc48 or MG63 cells. This result does not preclude an interaction *in vivo*, since other proteins which are known to interact with the IGFIR, such as Shc, cannot be detected in receptor immunoprecipitates. It is also possible that 14-3-3 proteins are true substrates of the receptor and dissociate from it after phosphorylation. In this model, phosphorylation would be expected to alter the binding of 14-3-3 to its downstream targets.

In summary, we have found that 14-3-3β and 14-3-3ζ bind selectively to the IGFIR in the yeast two-hybrid system *in vitro* and in insect cells overexpressing the receptor, and that these proteins are phosphorylated by the receptor in yeast and *in vitro*. In the yeast system the interaction maps to Ser-1283 in the C-terminus of the IGFIR and requires phosphorylation of this residue. These findings suggest that 14-3-3 proteins may play a role in IGFIR signal transduction and may contribute to the differences in IGF and insulin receptor signalling capacities.

We thank Patricia Aha, Elaine Hansen and Cheryl Terry for their technical assistance. We also thank the many investigators who provided reagents used in these studies. This work was supported by grants from the Genentech Foundation for Growth and Development and the National Institutes of Health to R.W.F.

REFERENCES

1 Jones, J. I. and Clemmons, D. R. (1995) Endocr. Rev. **16**, 3–34
2 LeRoith, D., Werner, H., Beitner-Johnson, D. and Roberts, C. T. (1995) Endocr. Rev. **16**, 143–163
3 Cheatham, B. and Kahn, C. R. (1995) Endocr. Rev. **16**, 117–142
4 Myers, Jr., M. G. and White, M. F. (1995) Trends Endocrinol. Metab. **6**, 209–215

5 Sun, X., Wang, L., Zhang, Y., Yenush, L., Myers, Jr., M., Glasheen, E., Lane, W., Pierce, J. and White, M. (1995) Nature (London) **377**, 173–177
6 Sasaoka, T., Rose, D. W., Jhun, B. H., Saltiel, A. R., Draznin, B. and Olefsky, J. M. (1994) J. Biol. Chem. **269**, 13689–13694
7 Lammers, R., Gray, A., Schlessinger, J. and Ullrich, A. (1989) EMBO J. **8**, 1369–1375
8 Miura, M., Surmacz, E., Burgaud, J. and Baserga, R. (1995) J. Biol. Chem. **270**, 22639–22644
9 Faria, T. N., Blakesley, V. A., Hisanori, K., Stannard, B., LeRoith, D. and Roberts, C. T. (1994) J. Biol. Chem. **269**, 13922–13928
10 Liu, D., Zong, C. and Wang, L. (1993) J. Virol. **67**, 6835–6840
11 Hongo, A., D'Ambrosio, C., Miura, M., Morrione, A. and Baserga, R. (1996) Oncogene **12**, 1231–1238
12 Surmacz, E., Sell, C., Swantek, J., Kato, H., Roberts, Jr., C., LeRoith, D. and Baserga, R. (1995) Exp. Cell Res. **218**, 370–380
13 Li, S., Resnicoff, M. and Baserga, R. (1996) J. Biol. Chem. **271**, 12254–12260
14 Gyuris, J. and Brent, R. (1987) in Current Protocols in Molecular Biology, (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K., eds.), pp. 13.14.1–13.14.17, John Wiley and Sons, New York
15 Aitken, A., Collinge, D. B., van Heusden, B. P. H., Isobe, T., Roseboom, P. H., Rosenfeld, G. and Soll, J. (1992) Trends Biochem. Sci. **17**, 498–501
16 Reuther, G. W., Fu, H., Cripe, L. D., Collier, R. J. and Pendergast, A. M. (1994) Science **266**, 129–133
17 Conklin, D. S., Galaktionov, K. and Beach, D. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 7892–7896
18 Fu, H., Xia, K., Pallas, D. C., Cui, C., Conroy, K., Narsimhan, R. P., Mamon, H., Collier, R. J. and Roberts, T. M. (1994) Science **266**, 126–129
19 Freed, E., Symons, M., Macdonald, S. G., McCormick, F. and Ruggieri, R. (1994) Science **265**, 1713–1716
20 Fantl, W. J., Muslin, A. J., Kikuchi, A., Martin, J. A., MacNicol, A. M., Gross, R. W. and Williams, L. T. (1994) Nature (London) **371**, 612–614
21 Li, S., Janosch, P., Tanji, M., Rosenfeld, G. C., Waymire, J. C., Mischak, H., Kolch, W. and Sedivy, J. M. (1995) EMBO J. **14**, 685–695
22 Michaud, N. R., Fabian, J. R., Mathes, K. D. and Morrison, D. K. (1995) Mol. Cell Biol. **15**, 3390–3397
23 Zha, J., Harada, H., Yang, E., Jockel, J. and Korsmeyer, S. J. (1996) Cell **87**, 619–628
24 Vincenz, C. and Dixit, V. M. (1996) J. Biol. Chem. **271**, 20029–20034
25 Kadowaki, T., Bevins, C. L., Cama, A., Ojamaa, K., Marcus-Sammuels, B., Kawowaki, H., Beitz, L., McKeon, C. and Taylor, S. I. (1988) Science **240**, 787–790
26 Dey, B., Frick, K., Lopaczynski, W., Nissley, S. and Furlanetto, R. (1996) Mol. Endocrinol. **10**, 631–641
27 Tartare, S., Mothe, I., Kowalski-Chauvel, A., Breittmayer, J., Ballotti, R. and Van Obberghen, E. (1994) J. Biol. Chem. **269**, 11449–11455
28 Blakesley, V., Kalebic, T., Helman, L., Stannard, B., Faria, T., Roberts, Jr., C. and LeRoith, D. (1996) Endocrinology (Baltimore) **137**, 410–417
29 Jones, D., Ley, S. and Aitken, A. (1995) FEBS Lett. **368**, 55–58
30 Liu, D., Blenkowska, J., Petosa, C., Collier, R., Fu, H. and Liddington, R. (1995) Nature (London) **376**, 191–194
31 Xiao, B., Smerdon, S., Jones, D., Dodson, G., Soneji, Y., Aitken, A. and Gamblin, S. (1995) Nature (London) **376**, 188–191
32 Muslin, A., Tanner, J., Allen, P. and Shaw, A. (1996) Cell **84**, 889–897
33 Dent, P., Jelinek, T., Morrison, D. K., Weber, M. J. and Sturgill, T. W. (1995) Science **268**, 1902–1905.
34 Braselmann, S. and McCormick, F. (1995) EMBO J. **14**, 4839–4848
35 Jacobs, S., Sahyoun, N., Saltiel, A. and Cuatrecasas, P. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 6211–6213

Received 21 April 1997/23 June 1997; accepted 30 June 1997