14-3-3 binding to the IGF-1 receptor is mediated by serine autophosphorylation[1]

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Abstract **The phosphoserine-binding 14-3-3 proteins have been implicated in playing a role in mitogenic and apoptotic signaling pathways. Binding of 14-3-3 proteins to phosphoserine residues in the C-terminus of the insulin-like growth factor-1 receptor (IGF-1R) has been described to occur in a variety of cell systems, but the kinase responsible for this serine phosphorylation has not been identified yet. Here we present evidence that the isolated dimeric insulin-like growth factor-1 receptor kinase domain (IGFKD) contains a dual specific (i.e. tyrosine/serine) kinase activity that mediates autophosphorylation of C-terminal serine residues in the enzyme. From the total phosphate incorporation of ~4 mol per mol kinase subunit, 1 mol accounts for serine phosphate. However, tyrosine autophosphorylation proceeds more rapidly than autophosphorylation of serine residues ($t_{1/2}$ ~1 min vs. $t_{1/2}$ ~5 min). Moreover, dot-blot and far-Western analyses reveal that serine autophosphorylation of IGFKD is sufficient to promote binding of 14-3-3 proteins in vitro. The proof that dual kinase activity of IGFKD is necessary and sufficient for 14-3-3 binding was obtained with an inactive kinase mutant that was phosphorylated on serine residues in a stoichiometric reaction with the catalytically active enzyme. Thus, the IGF-1R itself might be responsible for the serine autophosphorylation which leads to recognition of 14-3-3 proteins in vivo.**

Key words: Insulin-like growth factor-1 receptor; Dual kinase activity; Serine autophosphorylation; 14-3-3; Protein–protein interaction

1. Introduction

The insulin-like growth factor-1 receptor (IGF-1R) consists of two α-subunits and two membrane-spanning β-subunits that form a disulfide-linked $(\alpha\beta)_2$-homodimer [1]. The α-subunits are located extracellularly and contain the ligand binding domain; each of the intracellular β-subunits contain a protein tyrosine kinase domain. Binding of IGF-1 to the receptor is believed to result in conformational rearrangements within the β-subunits, leading to autophosphorylation of multiple tyrosine residues within the kinase domains, and subsequent kinase activation [2–4]. In addition to the hormone-induced tyrosine phosphorylation, IGF-1 stimulation has also been reported to result in serine phosphorylation of the receptor in vivo [5–7], but the biological role of this serine phosphorylation remains unknown. The phosphoserine-binding proteins of the 14-3-3 protein family have been implicated in playing roles in mitogenic and apoptotic signaling pathways [8–10]. Recently, phosphorylation of two serine residues in the C-terminus of the IGF-1R kinase (Ser^{1272} and/or Ser^{1283}) has been shown to result in binding of several isoforms of 14-3-3 proteins (β, ε, and ζ) to the receptor [11,12]. Phosphoserine-dependent 14-3-3 binding of the IGF-1R occurs in a variety of cell cultures, ranging from yeast to mammalian cell lines. However, the kinase responsible for this serine phosphorylation has not been identified yet. Moreover, the serine phosphorylation required for 14-3-3 binding was found to be strictly dependent on the kinase activity of the IGF-1R [12].

We have recently demonstrated that glutathione *S*-transferase (GST)-mediated dimerization of the soluble IGF-1R kinase domain (IGFKD) results in greatly enhanced catalytic activity in phosphorylation reactions [13]. Here we present evidence that the dimeric IGFKD contains a dual specific (i.e. tyrosine/serine) kinase activity that catalyzes autophosphorylation of C-terminal serine residues in the enzyme. Moreover, we show that serine autophosphorylation of the IGFKD is sufficient to promote binding of 14-3-3 proteins to the IGF-1R kinase in vitro.

2. Materials and methods

Plasmids for GST fusion proteins of human 14-3-3β and 14-3-3γ [14] were generous gifts from Dr. Michael B. Yaffe (Massachusetts Institute of Technology, Cambridge, MA, USA). The cDNA for human IGF-1R was kindly provided by Dr. Steen Gammeltoft (Glostrup Hospital, Glostrup, Denmark). [γ^{32}P]ATP (6000 Ci/mmol) was obtained from Amersham Pharmacia Biotech. Restriction endonucleases were purchased from MBI Fermentas and Roche, *Pfu* polymerase from Stratagene, ATP from Roche, thrombin from Pharmingen and cell culture reagents from Life Technologies. A polyclonal anti-GST antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA, anti-rabbit peroxidase-labeled antibody was purchased from Amersham Pharmacia Biotech. The enhanced chemiluminescence (ECL) detection kit was obtained from Pierce Rockford, IL, USA. Other reagents were obtained from commercial sources.

2.1. Construction of dimeric soluble IGF-1R kinases

Construction of a recombinant baculovirus for GST-IGFKD was

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[2] These authors contributed equally to this work.

Abbreviations: GST, glutathione *S*-transferase; IGF-1R, insulin-like growth factor-1 receptor; IGFKD, soluble insulin-like growth factor-1 receptor kinase domain; *Sf*9, *Spodoptera frugiperda*; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, polyvinyl difluoride; ECL, enhanced chemiluminescence

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described previously [13]. Construction of a truncation mutant, GST-IGFKDΔC, comprising the kinase domain from residues Val^{956}–Lys^{1256} will be published elsewhere (Baer et al., manuscript in preparation). GST-IGFKD-D/A, a mutant IGFKD in which the proposed catalytic base of the kinase, Asp-1105 [15], is replaced by alanine (D/A), was generated by site-directed mutagenesis using the polymerase chain reaction (PCR)-based Quick-Change method (Stratagene, La Jolla, CA, USA) and pUC-IGFR as template [16]. *Pfu* DNA-polymerase was used for 16–20 PCR amplification cycles according to the manufacturer's instructions. The mutagenesis primers (mismatches underlined) were: 5′-TCGTCCACAGAGCTCTTGCTGCCCGGAATTGC-3′ and 5′-CCGGGCAGCAAGAGCTCTGTGGACGAACTTATTGG-3′. The integrity of the cDNA was verified by automated DNA sequencing. The IGFKD-D/A cDNA was then subcloned into pAcG2T (Pharmingen), and the resulting construct was used to generate a recombinant baculovirus as described in [13].

2.2. Purification of recombinant IGFKDs

*Sf*9 cells containing GST-IGFKDs were lysed in 20 mM Tris–HCl, pH 7.5, 250 mM sucrose, 1% Triton X-100, 1 mM dithiothreitol (DTT) by mild sonication. Cleared lysates were used for affinity chromatography with glutathione–Sepharose (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The resulting eluates were passed over a ResourceQ anion exchange column, and eluted with a linear salt gradient (50 mM Tris–HCl, 100–300 mM NaCl, 1 mM DTT). Kinase-containing fractions were pooled, washed with 50 mM Tris–HCl, pH 7.5, 1 mM DTT and concentrated by ultrafiltration. For some experiments, affinity-purified ResourceQ-fractions of GST-IGFKD were further purified by size exclusion chromatography using a Superose 12 column (Amersham Pharmacia Biotech) equilibrated with 150 mM NaCl pH 7.5, 1 mM DTT. Kinase-containing fractions were pooled, washed with 50 mM Tris–HCl, pH 7.5, 1 mM DTT and concentrated by ultrafiltration. Monomeric receptor kinases (47 kDa) were generated by thrombin cleavage of the GST tag and purified by ResourceQ anion exchange column as described previously [13].

2.3. Phosphorylation reactions

All phosphorylation reactions were carried out at room temperature (22°C). Autophosphorylation reactions contained 50 mM Tris–acetate, pH 7.0, 30 mM $MgCl_2$, 1 mM DTT, and 1 mM [γ^{32}P]ATP. Unless indicated otherwise, the kinase concentration was 0.5–1 μM, and the reaction time was 30 min. Reactions were quenched by adding EDTA (50 mM, pH 7.5) or sodium dodecyl sulfate (SDS) sample buffer. For quantification of [^{32}P]phosphate incorporation, the proteins were separated by SDS–PAGE (polyacrylamide gel electrophoresis), localized by autoradiography and the radioactivity of the excised bands was determined by measurement of the Cerenkov radiation in a Beckman scintillation counter.

2.4. Phosphoamino acid analysis

^{32}P-labeled proteins were separated by SDS–PAGE, localized by autoradiography and excised from the gels. In-gel digestion of the proteins, hydrolysis, chromatography and electrophoresis of phosphoamino acids was performed as described in [16].

2.5. Dot-blot binding assay

2.5.1. Preparation of [^{32}P]-labeled dimeric IGFKDs. Purified GST kinases (IGFKD, IGFKDΔC) were autophosphorylated in the presence of [γ^{32}P]ATP as indicated. Kinase-inactive GST-tagged IGFKD-D/A was phosphorylated in the presence of [γ^{32}P]ATP by monomeric IGFKD at a 50:1 ratio for 10 min. GST kinases were re-purified by subsequent affinity chromatography with glutathione–Sepharose to remove unincorporated radioactive ATP and monomeric IGFKD.

2.5.2. Dot-blot. Affinity-purified GST-14-3-3γ was immobilized on polyvinyl difluoride (PVDF) membranes and the membrane sheets were blocked for 30 min in Tris-buffered saline (TBS; 50 mM Tris–HCl, pH 7.5, 150 mM NaCl), 1% bovine serum albumin (BSA) at 4°C. The membranes were then incubated for 2 h at 4°C with [^{32}P]-labeled IGFKDs in TBS, 0.1% BSA, washed 2×20 min in TBS, 1% BSA at 4°C and dried. The amount of bound [^{32}P]-labeled IGFKDs was determined by Phosphor-Imager analysis of the membranes.

2.6. Far-Western analysis

The GST-tagged IGFKD was autophosphorylated as described, and the GST tag was removed by thrombin cleavage. The monomeric kinase-inactive IGFKD-D/A was phosphorylated by GST-tagged IGFKD at a 1:1 ratio for 30 min. The samples were then subjected to SDS–PAGE and transferred to PVDF membranes. After blocking with TBS, 1% BSA, the membranes were incubated for 2 h at room temperature with 1 μM affinity-purified GST-14-3-3γ in TBS, 0.1% BSA, washed in TBS, 0.1% Tween-20, and incubated with a polyclonal anti-GST antibody from rabbit. After incubating the washed membranes with peroxidase-labeled anti-rabbit antibodies, membrane-bound GST-immunoreactivity was visualized by ECL according to the manufacturer's instructions.

2.7. Other procedures

Spodoptera frugiperda (*Sf*9) cells were maintained as described in [17]. Expression and purification of 14-3-3 proteins as GST fusion constructs from *Escherichia coli* was performed as described in [18]. Protein concentrations were determined by a modified method of Bradford [19]. SDS–PAGE was performed according to Laemmli [20]. Protein staining after SDS–PAGE was carried out by a modified colloidal Coomassie stain [21].

3. Results

3.1. Construction and purification of dimeric soluble IGF-1R kinases

The entire cytosolic domain of the IGF-1R (IGFKD) and a kinase-deficient mutant (IGFKD-D/A) were expressed as GST fusion proteins in *Sf*9 insect cells and purified by consecutive affinity chromatography and anion exchange chromatography as previously described [13] (see Section 2). Fig. 1 illustrates the course of purification for the ~76 kDa enzymes. Both constructs showed similar expression levels (3–5% of total protein) and similar yields (1–2 mg purified protein per 2×10^8 cells).

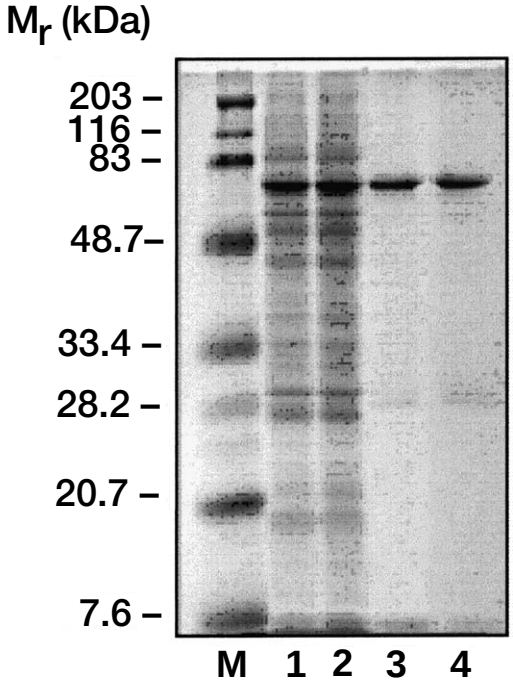

Fig. 1. Purification of GST-tagged IGF-1R kinases. Samples were resolved by SDS–PAGE and visualized by Coomassie staining. M, marker; 1 and 2, crude cell lysates of *Sf*9 cells expressing GST-IGFKD and GST-IGFKD-D/A; 3 and 4, affinity-purified GST-IGFKD and GST-IGFKD-D/A (76 kDa).

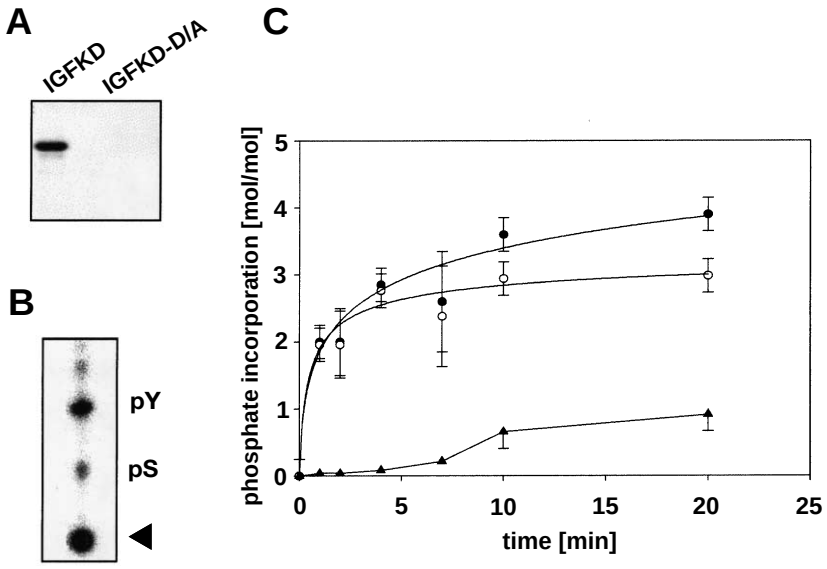

Fig. 2. Autophosphorylation of dimeric IGFKDs. A: Purified IGFKD and IGFKD-D/A were autophosphorylated in the presence of 1 mM [γ^{32}P]ATP as described in Section 2, subjected to SDS–PAGE and visualized by autoradiography. B: One-dimensional phosphoamino acid analysis of autophosphorylated IGFKD. pY, phosphotyrosine; pS, phosphoserine; ◀, origin. C: Time course of IGFKD autophosphorylation. Purified IGFKD (1 μM) was autophosphorylated in the presence of 1 mM [γ^{32}P]ATP for indicated times and resolved by SDS–PAGE. Total phosphate incorporation (●) was determined by Cerenkov counting of the excised bands. Phosphoamino acid analyses were performed as described in Section 2. ○, phosphotyrosine; ▲, phosphoserine.

3.2. Autophosphorylation of dimeric IGFKDs

The purified dimeric kinases, IGFKD and IGFKD-D/A (0.5–1 μM), were autophosphorylated for 30 min in the presence of [γ^{32}P]ATP and phosphate incorporation into the enzymes was determined after SDS–PAGE (see Section 2). As expected, autophosphorylation of IGFKD resulted in incorporation of labeled phosphate into the kinase, whereas no phosphate incorporation was observed for the kinase-inactive mutant (Fig. 2A). Similar to the previously described monomeric IGFKD [16], the dimeric enzyme showed autophosphorylation of tyrosine as well as of serine residues, respectively (Fig. 2B). However, whereas serine autophosphorylation of the monomeric kinase was observed only in the presence of poly-L-lysine [16], phosphoamino acid analyses of the dimeric IGFKD revealed serine phosphorylation even in the absence of the polycation. As shown in Fig. 2C, autophosphorylation

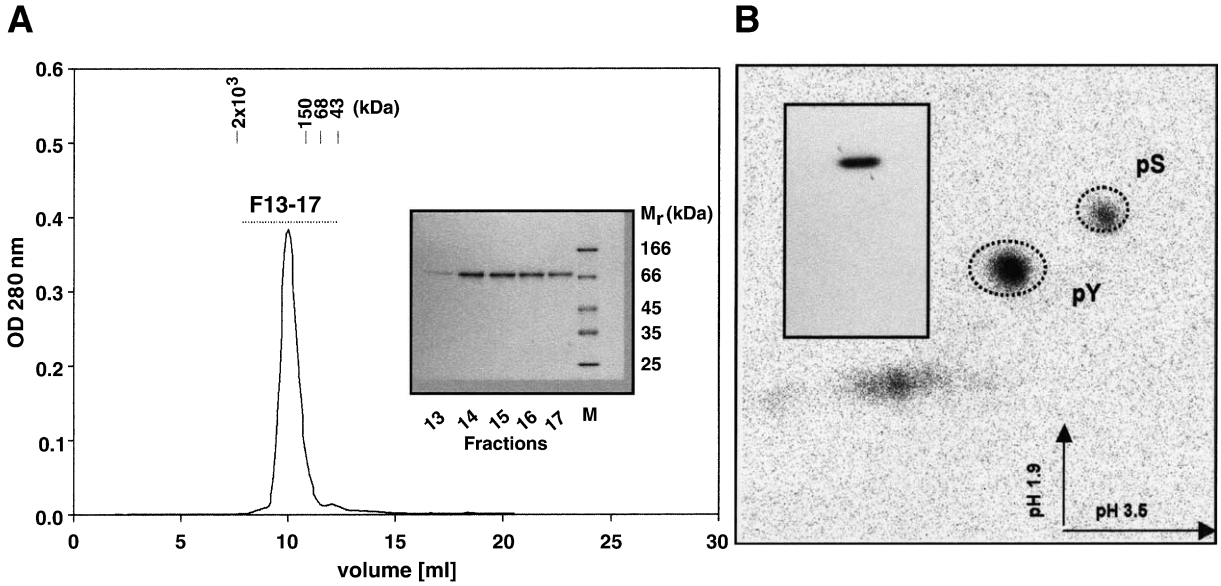

Fig. 3. Phosphoamino acid analysis of dimeric IGFKD after size exclusion chromatography. A: Preparative size exclusion chromatography of dimeric IGFKD. Affinity- and ion exchange-purified IGFKD (3 mg) was gel-filtrated on a Superose 12 column and kinase-containing fractions (bar with fraction numbers) were collected, resolved by SDS–PAGE and visualized by Coomassie staining (inset). Indicated are the elution positions of molecular weight standards: Blue Dextran [2×10^3 kDa], alcohol dehydrogenase [150 kDa], BSA [68 kDa] and ovalbumin [43 kDa]. A more precise determination of the M_r of the dimeric IGFKD has been described previously [13]. B: IGFKD-containing fractions were autophosphorylated in the presence of 1 mM [γ^{32}P]ATP as described in Section 2, subjected to SDS–PAGE and visualized by autoradiography (inset). Two-dimensional phosphoamino acid analysis of autophosphorylated IGFKD after size exclusion chromatography. pY, phosphotyrosine; pS, phosphoserine. Inset: Autoradiography of autophosphorylated IGFKD.

of the dimeric IGFKD resulted in incorporation of ~4 mol phosphate per mol of kinase subunit after 20 min reaction time. Even though tyrosine autophosphorylation proceeded considerably more rapidly than phosphorylation of serine residues ($t_{1/2}$ ~1 min vs. $t_{1/2}$ ~5 min), the latter resulted in stoichiometric phosphorylation of 1 mol serine phosphate per mol of kinase subunit (Fig. 2C).

In a recent report, Lopaczynski et al. [22] have described a serine/threonine kinase activity from *Sf*9 insect cells that co-purified with a soluble IGF-1R kinase in nickel chelate chromatography, but could be separated from the tyrosine kinase activity by size exclusion chromatography. Conversely, the observed serine kinase activity of the IGFKD described here appears to be strictly dependent on the enzymatic activity of the receptor kinase itself, and could not be separated from the IGFKD by Superose 12 size exclusion chromatography of the affinity- and ion exchange-purified enzyme (Fig. 3).

3.3. *In vitro interaction of 14-3-3 and IGFKDs*

Several isoforms of the 14-3-3 phosphoserine adaptor proteins have been reported to bind to the IGF-1R, implicating a possible role of this interaction in IGF-1 signaling. As described previously, the interaction between 14-3-3 proteins and the IGF-1R requires receptor-kinase activity and maps to two phosphoserine residues in the C-terminus of the enzyme, pSer^{1272} and/or pSer^{1283}. However, the serine kinase responsible for phosphorylation of Ser^{1272} and/or Ser^{1283} remains unknown. Thus, we have investigated the question whether IGFKD serine autophosphorylation is sufficient to promote binding of 14-3-3 proteins to the kinase in vitro.

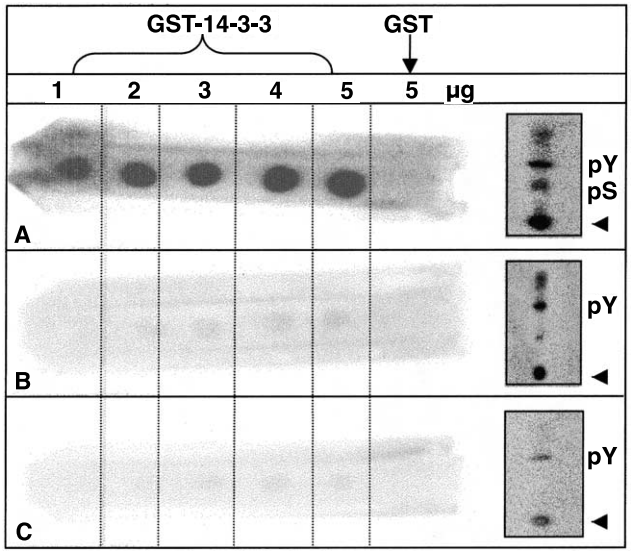

Fig. 4. In vitro interaction of GST-14-3-3γ and dimeric ^{32}P-labeled IGFKDs. Purified GST-14-3-3γ (1–5 µg) was immobilized on PVDF membranes and incubated with ^{32}P-labeled IGFKD constructs. After several washes, the 14-3-3-bound radioactivity was detected by autoradiography of the membrane strips as described in Section 2. ^{32}P-IGFKDs: A, autophosphorylated IGFKD; B, autophosphorylated truncation mutant IGFKDΔC lacking amino acid residues Leu^{1257}–Cys^{1337}; C, kinase-inactive GST-tagged IGFKD-D/A phosphorylated by active monomeric IGFKD. The inactive kinase was phosphorylated using a 50:1 substrate:enzyme ratio for 10 min and then repurified by glutathione affinity chromatography to remove the non-tagged active kinase (see Section 2). Insets: One-dimensional phosphoamino acid analyses of the corresponding ^{32}P-IGFKDs. pY, phosphotyrosine; pS, phosphoserine; ◄, origin.

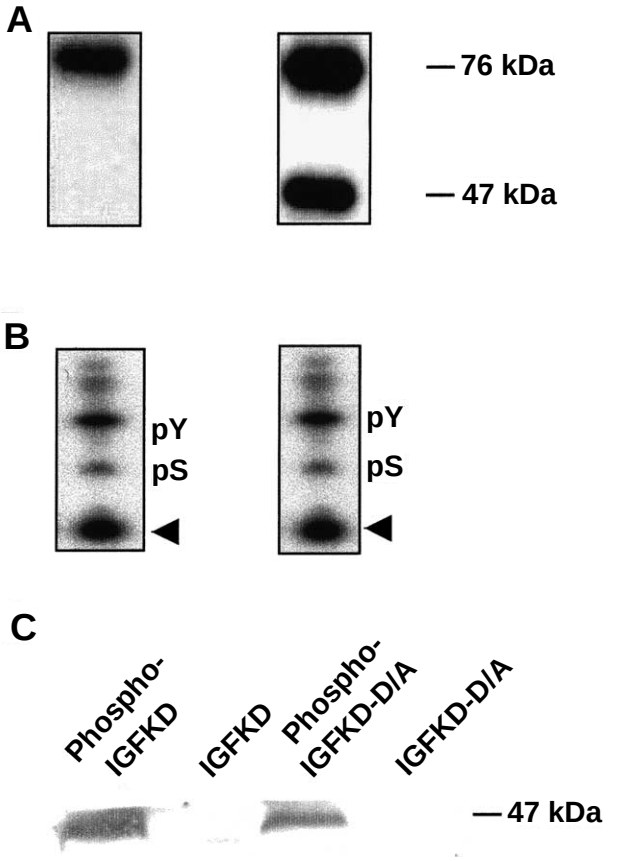

Fig. 5. Dual kinase activity of IGFKD itself is responsible for 14-3-3 binding in vitro. A: Phosphorylation of dimeric IGFKD in the absence (left) and presence (right) of equimolar amounts of kinase-inactive monomeric IGFKD-D/A. Phosphorylation reactions were carried out for 30 min in the presence of 1 mM [γ^{32}P]ATP (specific activity ranging from 80 to 250 cpm/pmol). Samples were subjected to SDS–PAGE and visualized by autoradiography. B: One-dimensional phosphoamino acid analyses of the corresponding dimeric IGFKD (left) and monomeric IGFKD-D/A (right). pY, phosphotyrosine; pS, phosphoserine; ◄, origin. C: Far-Western analysis of IGFKD/14-3-3 interaction. The monomeric 47 kDa kinases (phosphorylated: phospho-IGFKD, phospho-IGFKD-D/A; non-phosphorylated: IGFKD, IGFKD-D/A) were prepared by enzymatic removal of the GST tag as described in Section 2, subjected to SDS–PAGE and transferred to PVDF membrane. After probing the membrane with 1 µM GST-14-3-3γ, kinase-bound 14-3-3 proteins were detected by ECL Western blotting using an anti-GST-antibody (see Section 2).

In the experiment shown in Fig. 4, purified GST-14-3-3γ was immobilized on PVDF membranes and then incubated with purified [^{32}P]-labeled dimeric IGFKD constructs. After several washes, the 14-3-3-bound radioactivity was then detected by autoradiography of the membrane strips (see Section 2). As illustrated in Fig. 4A, serine autophosphorylated wild-type IGFKD interacted with immobilized 14-3-3γ but not with immobilized GST. Moreover, no 14-3-3γ binding was observed with an autophosphorylated, truncated IGFKD mutant (IGFKDΔC; Baer et al., manuscript in preparation) lacking the C-terminal amino acid residues Leu^{1257}–Cys^{1337} (Fig. 4B). Notably, phosphoamino acid analysis revealed that autophosphorylation of IGFKDΔC did result in phosphorylation of tyrosine residues only (Fig. 4).

Even though the kinase-deficient mutant, IGFKD-D/A, showed no detectable kinase activity in phosphorylation reac-

tions (Fig. 2A), the protein served as a substrate for the kinase-active IGFKD. Short-term phosphorylation (10 min) of IGFKD-D/A with a low ratio of active to inactive enzyme (1:50) resulted in almost exclusive tyrosine phosphorylation of the inactive kinase (Fig. 4C). As expected, the tyrosine-phosphorylated IGFKD-D/A did not bind to immobilized 14-3-3γ (Fig. 4C). Similar results were obtained for the β-isoform of 14-3-3 (data not shown).

3.4. Autophosphorylation of C-terminal serine residues is sufficient for 14-3-3 binding in vitro

As demonstrated in Fig. 4C, substrate phosphorylation of IGFKD-D/A using a catalytic amount (1:50) of active wild-type IGFKD led to phosphorylation of tyrosine residues in the kinase mutant. In contrast, long-term phosphorylation (30 min) of IGFKD-D/A with a high ratio of active to inactive enzyme (1:1) resulted in substrate phosphorylation of serine as well as tyrosine residues, respectively (Fig. 5A,B) (cf. [23]). In fact, the phosphoamino acid composition of IGFKD-D/A phosphorylated as a substrate under those conditions was indistinguishable from that of the wild-type kinase autophosphorylated under standard conditions (Fig. 5B).

To prove that the IGFKD itself is the unknown kinase that is responsible for the serine phosphorylation (and thus binding to 14-3-3), we have phosphorylated IGFKD-D/A as a substrate by using wild-type dimeric IGFKD and then monitored 14-3-3 binding by far-Western blot analysis. As controls we used active autophosphorylated and non-phosphorylated dimeric IGFKD. The GST tags of the kinases were cleaved off, and the proteins were separated by SDS–PAGE. After transfer to PVDF membranes and incubation of the membrane sheets with GST-14-3-3, protein–protein interactions were detected via an anti-GST antibody as described in Section 2.

According to Fig. 5C, 14-3-3 did not interact with wild-type IGFKD purified from insect cells. However, 14-3-3 binding occurred after autophosphorylation of the kinase. Likewise, 14-3-3 did not bind to the purified inactive kinase mutant, whereas complex formation occurred when IGFKD-D/A was phosphorylated on serine and tyrosine residues by the active wild-type kinase (Fig. 5C). Similar results were obtained for the β-isoform of 14-3-3 (data not shown).

4. Discussion

Both serine phosphorylation and phosphoserine-dependent 14-3-3 binding of the IGF-1R kinase have been described to occur in a variety of cell systems, such as yeast cells, *Sf*9 insect cells, and several non-related mammalian cell lines [11,12]. Even though the binding sites for 14-3-3 proteins in the IGF-1R kinase have been mapped to two particular phosphoserine residues in the C-terminus of the enzyme ($pSer^{1272/1283}$; [11,12]), the kinase responsible for this serine phosphorylation has not been identified yet. However, the serine phosphorylation required for 14-3-3 binding has been found to be strictly dependent on the kinase activity of the IGF-1R kinase [12]. Thus, phosphorylation of those serine residues might be the result of an activity from a ubiquitously expressed protein serine kinase that appears to be expressed in all cell types tested and, in addition, is somehow activated by the IGF-1R kinase. However, the experimental evidence presented in this report strongly suggests that the IGF-1R kinase contains a dual specific (i.e. tyrosine/serine) kinase activity that is involved in creating docking sites for 14-3-3 proteins.

Previously, we have reported that the cytoplasmic domain of the IGF-1R purified from baculovirus-infected *Sf*9 cells by sequential ion exchange and hydrophobic interaction chromatography showed autophosphorylation of both tyrosine and serine residues in vitro [16]. Interestingly, the observed serine phosphorylation of the monomeric enzyme occurred only with the fully activated receptor kinase, i.e. in the presence of the cationic compound poly-L-lysine that is known to promote kinase oligomerization [24]. In a more recent report, we showed that GST-mediated kinase dimerization of IGFKD results in greatly enhanced catalytic activity in both auto- and substrate phosphorylation reactions, thereby eliminating the stimulatory effect of poly-L-lysine [13]. Accordingly, besides an increased activity of the tyrosine kinase, here we demonstrate that the affinity-purified dimeric IGFKD showed serine phosphorylation even in the absence of the polycation, resulting in stoichiometric serine phosphorylation (1 mol phosphate/mol kinase subunit) similar to that observed for the monomeric kinase in the presence of poly-L-lysine [16]. Because in autophosphorylation reactions the kinase-inactive mutant IGFKD-D/A did not show any incorporation of phosphates, it is evident that IGFKD serine phosphorylation in vitro requires an intact active site of the enzyme. Thus, the observed serine phosphorylation of the purified dimeric IGFKD is best explained to occur via an intrinsic serine kinase activity of the IGF-1R kinase.

In a recent report, Lopaczynski et al. [22] have described a serine kinase from *Sf*9 insect cells that co-purified with a monomeric histidine-tagged IGF-1R kinase, leading to the phosphorylation of unspecified serine/threonine residues in the enzyme. However, whereas the *Sf*9-kinase could be removed from IGF-1R kinase preparations by a simple gel-filtration step, we were unable to separate the serine kinase activity from the affinity-purified dimeric IGFKD by gel filtration and ion exchange chromatography (Fig. 3 and data not shown). Moreover, under autophosphorylation conditions described by Lopaczynski et al. [22], we did not detect any serine phosphorylation of the monomeric non-tagged IGFKD [16], indicating that this *Sf*9-kinase may co-purify due to the metal-chelate chromatography of the histidine-tagged receptor kinase.

Consistent with previous reports from studies in vivo [11,12,16], we show that in vitro interaction of the two purified proteins, 14-3-3 and IGFKD, depends on both the presence of an active IGF-1R kinase and the presence of phosphoserine residues in the C-terminal domain of the enzyme. The purified, autophosphorylated dimeric IGFKD showed specific binding to immobilized 14-3-3 (Fig. 4), and conversely, soluble 14-3-3 showed binding to the immobilized, autophosphorylated IGFKD (Fig. 5). In contrast, a kinase-inactive mutant as well as the non-phosphorylated wild-type kinase did not bind 14-3-3 (Fig. 5). Likewise, autophosphorylation of the kinase-active truncation mutant IGFKDΔC resulted in phosphorylation of tyrosine residues only, and no interaction with 14-3-3 was observed. To establish whether the C-terminal serine phosphorylation of the IGFKD is sufficient for 14-3-3 binding, the inactive IGFKD-D/A mutant was phosphorylated by the wild-type IGFKD, separated by SDS–PAGE and then assayed for 14-3-3 binding in far-Western blots. Interestingly, whereas tyrosine phosphorylation of

IGFKD-D/A occurred even at low enzyme-to-substrate ratios, significant serine phosphorylation of IGFKD-D/A was observed only at equimolar amounts of kinase and substrate (Fig. 5B) (cf. [23]). As a result, only the serine-phosphorylated kinase showed binding of 14-3-3 (Fig. 5C). Thus, we conclude that autophosphorylation of serine residues in the C-terminus of the enzyme (presumably $Ser^{1272/1283}$) generates the docking sites required for the interaction of 14-3-3 proteins and the IGF-1R kinase.

The biological role of the 14-3-3/IGF-1R kinase interaction remains to be clarified. 14-3-3 proteins have been implicated in the regulation of a variety of cellular events including neurotransmitter biosynthesis, vesicular trafficking, cell-cycle progression, and protection from apoptosis [8–10]. Our finding that the dual kinase activity of the activated IGF-1R kinase is responsible for the in vitro generation of docking sites required for 14-3-3 binding suggests that this interaction may be involved in IGF-1 signaling. Certainly, we cannot entirely rule out a possible involvement of an exogenous serine kinase in the creation of 14-3-3 binding sites of the IGF-1R in vivo. However, our study presents strong evidence that 14-3-3 binding to the IGFKD is the result of an autocatalytic event.

Interestingly, time course experiments in vitro indicate that IGFKD serine autophosphorylation is delayed with respect to the tyrosine autophosphorylation, where the maximum of phosphoserine content is reached significantly later than maximum phosphotyrosine content. However, the biological consequence of this observation with respect to IGF-1 signaling remains to be determined. Noticeably, 14-3-3 binding to the receptor kinase in vitro did not affect the catalytic activity of the phosphotransferase towards model substrates (data not shown). Nevertheless, the interaction of IGF-1Rs and 14-3-3 proteins in vivo might be involved in modulating a variety of processes such as the recruitment of IGF-1 signaling components to the receptor. Thus, further studies in vivo are required to elucidate the specific biological function of this interaction in IGF-1 target tissues.

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