GIPC Participates in G Protein Signaling Downstream of Insulin-like Growth Factor 1 Receptor*

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Several recent studies have demonstrated that insulin-like growth factor (IGF)-1-induced mitogen-activated protein kinase (MAP kinase) activation is abolished by pertussis toxin, suggesting that trimeric G proteins of the G, class are novel cellular targets of the IGF-1 signaling pathway. We report here that the intracellular domain of the Xenopus IGF-1 receptor is capable of binding to the Xenopus homolog of mammalian GIPC, a PDZ domain-containing protein previously identified as a binding partner of G,-specific GAP (RGS-GAIP). Binding of xGIPC to xIGF-1 receptor is independent of the kinase activity of the receptor and appears to require the PDZ domain of xGIPC. Injection of two C-terminal truncation mutants that retained the PDZ domain blocked IGF-1-induced Xenopus MAP kinase activation and oocyte maturation. While full-length xGIPC injection did not significantly alter insulin response, it greatly enhanced human RGS-GAIP in stimulating the insulin response in frog oocytes. This represents the first demonstration that GIPC-RGS-GAIP complex acts positively in IGF-1 receptor signal transduction.

Insulin-like growth factor 1 (IGF-1) exerts its biological roles by activating the intrinsic protein tyrosine kinase activity of the IGF-1 receptor. The activated IGF-1 receptor autophosphorylates its cytoplasmic domain and phosphorylates insulin receptor substrate 1 (IRS-1) and many other protein substrates. Phosphorylation of these protein substrates allows changes in multiple intracellular signaling pathways including the Ras-Raf-MAP kinase pathway and the phosphatidylinositol 3-kinase (PI 3-kinase)/Akt pathway (1). However, these same changes in multiple intracellular signaling pathways including the Ras-Raf-MAP kinase pathway and the phosphatidylinositol 3-kinase (PI 3-kinase)/Akt pathway (1). However, these same changes in multiple intracellular signaling pathways including the Ras-Raf-MAP kinase pathway and the phosphatidylinositol.
between the GAL4 DNA-binding domain and the xIGF-1R cytoplasmic domain. The kinase-deficient mutant of xIGF-1R (xIGF-1R<sub>K ΔΔ</sub>) in pAS2 was constructed by the two-step PCR procedure (8) using the same forward and reverse primers (above) in combination with the following internal primers changing the catalytically essential Lys-1029 to Ala: forward primer, 5′-GAT GCC ATA GCC AGC GTC AAC G-3′; reverse primer, 5′-C GAT GCC CGT CGT TAT GCC AAC TTT C-3′. To create xIGF-1R<sub>ΔΔ</sub> and xIGF-1R<sub>KΔΔ</sub> for transfection or mRNA synthesis, the same PCR products were digested with EcoRI and ligated into pcS2+Hyg previously digested with EcoRI.

Upon sequence analysis, YA 5-2, the clone identified in the yeast two-hybrid screen, was found to have its own open reading frame (ORF) (xGIPC) inserted in a reverse orientation relative to the GAL4 activation domain (AD). To clone xGIPC in-frame with the GAL4AD, a 5′ PCR primer was designed to amplify the coding region (5′-TAT GAA TTC A AT GCC CAC CCC GCA TGA C-3′). This primer, along with a pGAD10 vector (two-hybrid)-specific primer, was used to amplify the ORF. The PCR product was digested with EcoRI and ligated into the pGAD10 previously digested with EcoRI. To create an xGIPC for transfection and mRNA synthesis, the same PCR product was digested with EcoRI and treated with Klenow to generate a 5′ EcoRI site. pcS2+Hyg was modified from pcS2+ (9) as follows: Two complementary oligonucleotides were made to code for the hemagglutinin (HA) epitope (NVVDYVPDYA) with a translation initiation codon and the cohesive BamHI ends. The sequences of the two oligonucleotides were as follows: forward, 5′-GAT CCA CCA TGG ACC CAT CAG ATG TTC CAG ATT GCC CCA TTC CTA G-3′; reverse, 5′-GAT CCA TGG AAG CTT CCA TG-3′. The oligonucleotides were annealed to each other and then directly ligated into pcS2+ vector previously digested with BamHI.

Subclones of xGIPC for mapping the binding region of xGIPC and for injection into oocytes were similarly generated by PCR using primers containing an EcoRI site. The PCR fragments were ligated into either pGAD10 vector or pcS2+Hyg vector. The resulting clones generated were fusion proteins with the GAL4AD or a HA tag, respectively. For the sake of brevity, only the amino acids comprising the various constructs are described in the figures. To reconstitute full-length xGIPC, we PCR-amplified xGIPC (1–320) with a reversed primer encoding 11 extra amino acids (GAIGDAKQGRF) derived from the sequence of the Xenopus expressed sequence tag clone 10.

The human GAIP coding sequence was PCR-amplified from a human brain cDNA library (a gift of J. K. Ngsee) using the following primers: forward primer, 5′-TAT GAA TTC A AT GCC CAC CCC GCA TGA C-3′ and reverse primer, 5′-TAT GAA TTC A AT GCC CAC CCC GCA TGA C-3′. The PCR product was digested with EcoRI and ligated at the EcoRI site of pcS2+ or pcS2+MT (9), resulting in the expression of an untagged or Myc-tagged version of human GAIP. The identity of the sequence was confirmed by DNA sequence determination.

**Yeast Two-hybrid Analysis**—These procedures are essentially the same as described in the *Yeast Protocol Handbook* provided by CLONTECH. *Saccharomyces cerevisiae* strain Y190 was co-transformed with the bait plasmid (pAS-xIGF-1R<sub>ΔΔ</sub>) and a X. laevis oocyte cDNA library constructed in the pGAD10 vector (CLONTECH). Transforms were plated on 150-mm synthetic dropout medium (Yeast Protocol Handbook, CLONTECH). His/Leu/Trp/β-amino-1,2,4-triazole (50 mm) plates and incubated at 30°C until colonies reached 1–2 mm. β-Galactosidase assays for the detection of potential positive clones were performed according to the protocols supplied by CLONTECH. Potential positive clones were grown in liquid synthetic dropout medium/Leu<sup>-</sup> and the pGAD10 library plasmid was isolated and transformed into *Escherichia coli* strain MH6 by electroporation. The transformants were plated on M9 media (11 mm Na<sub>2</sub>HPO<sub>4</sub> 22 mm KH<sub>2</sub>PO<sub>4</sub> 5.5 mm NaCl 19 mm NH<sub>4</sub>Cl 1 mm MgSO<sub>4</sub> 100 μm CaCl<sub>2</sub> 2 mg/ml thiamine, 20 mg/liter uracil, 100 μm ampicillin) and incubated at 37°C for 20 h. The isolated plasmid was used in co-transformation assays with the original bait plated on synthetic dropout medium/Leu<sup>-</sup> to transform *Escherichia coli*. Colonies were tested for filter-based β-galactosidase activity. Yeast protein extraction for immunoblotting was carried out using the method described by CLONTECH.

**Polyclonal Antibodies against xGIPC**—An internal HindIII fragment of xGIPC, encoding amino acids 216–305, was excised and inserted into pGEX-KT (11). Glutathione S-transferase fusion proteins were induced and purified by binding to glutathione-agarose beads. Immunization of rabbits was carried out according to Harlow and Lane (12). Immune sera were used without further processing.

**Co-immunoprecipitation Experiments**—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 50 units/ml penicillin-streptomycin, and 2 ml/liter Fungizone (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Protein Kinase Assays**—Protein kinase assays were performed on immunoprecipitated xIGF-1R<sub>ΔΔ</sub> or xIGF-1R<sub>KΔΔ</sub>. Briefly, immunoprecipitation procedures were carried out as above with the exception that the last wash step was done with kinase buffer (50 mM Hepes, pH 6.9, 1 mM EDTA, 0.1% Triton X-100). Following centrifugation, 25 μl of kinase buffer were added, and the reaction was started with the addition of 1 μg/ml of anti-Myc- or anti-HA-Sepharose beads (14). Lysates were incubated at 4°C with rocking for 2 h. Immunocomplexes were washed five times with PBS-Lysis buffer; after washing, 20 μl of 2× Laemmli sample buffer was added, and the samples were separated using SDS-PAGE. The proteins were transferred to a nitrocellulose membrane for Western blotting.

**Co-immunoprecipitation experiments** using frog oocyte extracts were performed essentially the same way. Oocytes were injected with the various mRNA and incubated overnight. Extracts were prepared in PBS-Lysis buffer (10 μl/oocyte) and subjected to immunoprecipitation (200–400 μl of extract with 5 μl of either preimmune serum or anti-xGIPC immune serum).

**RESULTS**

We inserted the cytoplasmic domain of xIGF-1 receptor (7) into the bait vector pAS2. The resultant plasmid (pAS-xIGF-

![Fig. 1. xIGF-1R<sub>ΔΔ</sub> is active in yeast. Extracts from control yeast or yeast transformed with pAS-xIGF-1R<sub>ΔΔ</sub> or with pAS-xIGF-1R<sub>KΔΔ</sub> were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Arrows indicate GAL4 fusion proteins containing xIGF-1R<sub>ΔΔ</sub> or xIGF-1R<sub>KΔΔ</sub>, pY, phosphotyrosine.](http://www.jbc.org/figs/)

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Rcyto) encodes an HA epitope tag followed by the DNA-binding domain of GAL4 and then by the xIGF-1 receptor cytoplasmic domain. We also constructed similar bait (pAS-xIGF-1RKAcyto) with a single point mutation changing the catalytically essential lysine 1029 to alanine. Transforming yeast with each plasmid resulted in expression of the fusion protein with the anticipated size as indicated by both anti-xIGF-1 receptor blots and anti-HA blots (Fig. 1). As expected, pAS-xIGF-1Rcyto was phosphorylated on tyrosine, whereas tyrosine phosphorylation of pAS-xIGF-1RKAcyto was undetectable.

Using pAS-xIGF-1Rcyto as bait, we screened an oocyte cDNA library constructed in the pGAD10 vector (encoding the GAL4AD) (by CLONTECH). One particular clone, YA5-2, caught our attention since it contained an ORF highly homologous to a mammalian gene, GIPC, previously identified as a binding partner for a Gi-specific GTPase-activating protein (RGS-GAIP or GAIP) (16). We thought that perhaps GIPC might provide a functional link between IGF-1 receptor and Gi protein signaling. However, the ORF of 320 amino acids, which included a candidate translation start site but lacked a translation termination codon, was inserted in pGAD10 with a reverse orientation. Therefore xGIPC could not have been made as part of a GAL4AD fusion protein. This clone was therefore initially discarded as a "false positive" until one of us (R. A. B.) found, in searching the literature, that the ADH1 termination sequences actually contain functional promoter sequences capable of initiating transcription opposite to the ADH promoter in pGAD10 (17). Consequently xGIPC could have been expressed in yeast from the promoter contained within the ADH1 termination sequences as a nonfusion protein.

We carried out further yeast two-hybrid assays to confirm that xGIPC was indeed capable of interacting with xIGF-1Rcyto and attempted to define the region of xGIPC responsible for the interaction. xGIPC-(1–320), the PDZ domain, or the arbitrarily defined N or C terminus (Fig. 3A) were PCR-amplified and inserted into pGAD10. Co-transformation of each plasmid with xIGF-1Rcyto, xIGF-1RKAcyto, or a control plasmid xIRS-1-(3–500) (13) was carried out. We found that GALAD-xGIPC-(1–320), like YA 5-2, interacted strongly with xIGF-1Rcyto. Both YA 5-2 and GAL4AD-xGIPC-(1–320) also interacted strongly with the kinase-deficient mutant xIGF-1RKAcyto. Neither was able to interact with xIRS-1-(3–500), cloned in the same vector (13), or the vector alone (not shown). A deletion mutant (xGIPCN-PDZ) missing the C terminus behaved very similarly to YA 5-2 or xGIPC-(1–320) (Fig. 3B). However, none of the three regions of xGIPC (the N terminus, the PDZ domain, or the C

**Fig. 2. Sequence comparison among mouse, rat, human, and Xenopus GIPC.** GIPC amino acid sequences from the various species were aligned using the web-available CLUSTAL W Multiple Sequence Alignment Program. The stars (*) indicate matches, whereas the colon (:) and period (.) indicate strong and weaker conservative changes, respectively. The PDZ domain is underlined. Numbers indicate positions of the amino acids in xGIPC. The C-terminal 11 amino acids (boldface) were derived from a Xenopus expressed sequence tag clone (GenBank™ accession no. AW646185).
terminus), when expressed separately with GAL4AD, were able to interact with xIGF-1Rcyto or xIGF-1RKAcyto (Fig. 3B).

We decided to carry out binding experiments in transiently transfected COS-7 cells following an unsuccessful attempt to demonstrate co-immunoprecipitation of endogenous xIGF-1 receptor and xGIPC in frog oocytes (see below). To facilitate these experiments, we subcloned xIGF-1Rcyto or xIGF-1RKAcyto into pCS2/H11001Myc (9) and xGIPC or its truncation mutants (Fig. 3A) into pCS2/H11001HA. Transient transfection of Myc-xIGF-1Rcyto or Myc-xIGF-1RKAcyto resulted in similar levels of the two proteins, recognized by anti-Myc (Fig. 4A, lower panel). Immuno-precipitation followed by an in vitro kinase assay confirmed that Myc-xIGF-1RKAcyto was indeed deficient in catalytic activity (Fig. 4A, upper panel). Co-transfection of each plasmid with HA-xGIPC followed by co-immunoprecipitation experiments clearly indicated that xGIPC was able to bind both the kinase-active and kinase-deficient cytoplasmic domain of the xIGF-1 receptor (Fig. 4B, lanes 7 and 8). Lanes 5 and 6 in Fig. 4B represent HA immunoprecipitation from extracts derived from cells expressing only the Myc-tagged proteins, indicating that the co-immunoprecipitation observed in Fig. 4B, lanes 7 and 8, were not due to the overexpressed Myc fusion proteins being "pulled down" by anti-HA antibodies nonspecifically.

We wished to determine whether overexpression of xGIPC (1–320) and the various truncation mutants had any effect on IGF-1 receptor signaling in frog oocytes. mRNA encoding green fluorescent protein (18) (used as a control), HA-xGIPC-(1–320), or HA-xGIPCPDZ were injected into immature oocytes. Following overnight incubation to allow translation and accumulation of the corresponding proteins, oocytes were incubated with insulin (200 or 500 nM). We have previously shown that these concentrations of insulin activate the endogenous xIGF-1 receptor (7, 19) and ultimately cause oocyte maturation, indicated by, among other criteria, xMAP kinase activation and GVBD. Fig. 5A shows that injection of the PDZ domain mRNA had no significant effect (compared with similar amount of green fluorescent protein mRNA or water) on insulin-induced GVBD. In contrast, injection of xGIPC-(1–320) mRNA severely reduced the ability of insulin to induce GVBD. Fig. 5B shows a typical experiment where we analyzed xMAP kinase phosphorylation (indicative of activation (20, 21)) following scoring oocytes for GVBD (Fig. 5A). Again, xGIPC-(1–320), but not the PDZ domain in isolation, significantly reduced the ability of insulin to activate xMAP kinase phosphorylation (activation). Immunoblotting using anti-HA antibodies demonstrated that both xGIPC-(1–320) and HA-xGIPCPDZ were expressed and accumulated in oocytes (Fig. 5A, inset). Similarly, injection of mRNA encoding either the N terminus or the C terminus of xGIPC did not significantly change insulin-induced xMAP kinase phosphorylation (Fig. 5C) or GVBD (not shown).

A recently deposited Xenopus expressed sequence tag clone (GenBank™ accession no. AW646188) (10) apparently contains the missing 3′-end of our xGIPC sequence. This clone contains 240 bp of coding sequence that is identical to the corresponding region of xGIPC (with the exception, of course, of the 11 extra codons that are missing from our clone). Full-length xGIPC was therefore generated by PCR amplification of xGIPC-(1–320) with a 3′-primer that contained 11 extra codons
(GAIGDAKQGRF). To our surprise, the full-length xGIPC, unlike xGIPC-(1–320), did not inhibit insulin-induced oocyte maturation assayed by three different criteria: GVBD (Fig. 6A), MPF activation (Fig. 6B), and xMAP kinase phosphorylation (Fig. 6C). In contrast a C-terminal deletion mutant (xGIPC-(1–320)), which was capable of binding xIGF-1 receptor cytoplasmic domain in the yeast assays (Fig. 3B), significantly reduced insulin-induced oocyte maturation. Immunoblotting indicated that xGIPC and xGIPC-(1–320) were expressed at similar levels in all three forms of mRNA-derived xGIPC (Fig. 7C). The inhibitory effect of xGIPC-(1–320) was reminiscent of that of xGIPC-(1–320) (Fig. 5A and B).

To further investigate the mechanism of xGIPC in insulin signaling, we explored the possible functional interaction between xGIPC and GAIP in frog oocytes. Human GAIP (hGAIP) cDNA was PCR-amplified and expressed with or without an N-terminal Myc tag. Our preliminary injection experiments indicated that hGAIP significantly accelerated insulin-induced GVBD (not shown). We used a concentration of insulin (50 nM) that was not sufficient to induce oocyte maturation in most batches of frog oocytes. Under these conditions, injection of hGAIP mRNA (either untagged or Myc-tagged) resulted in oocyte maturation in a significant percentage of oocytes (Fig. 7, A and B). Although xGIPC alone had little effect, it greatly enhanced the ability of hGAIP in stimulating insulin-induced oocyte maturation (Fig. 7, A and B). To confirm that xGIPC bound hGAIP in frog oocytes, we performed co-immunoprecipitation experiments. Oocytes were injected with Myc-hGAIP alone or together with HA-xGIPC, HA-xGIPC-(1–320), or HA-xGIPC-N-PDZ. Immunoprecipitation with antibodies against xGIPC pulled down both endogenous xGIPC (not shown) and all three forms of mRNA-derived xGIPC (Fig. 7C, lower panel). Co-immunoprecipitation of Myc-hGAIP with the endogenous xGIPC was evident (Fig. 7C, lane 5 compared with lane 1, which represents preimmune control). The amounts of Myc-hGAIP co-precipitated in xGIPC immunoprecipitations increased significantly in all three groups of oocytes that had been injected with the various xGIPC mRNAs (Fig. 7C, lanes 6–8 as compared with lane 5). These results clearly indicated the ability of all three forms of xGIPC to bind Myc-hGAIP. As would be expected, nonspecific binding of Myc-GAIP in preimmune immunoprecipitations (Fig. 7C, lanes 1–4) did not change regardless of whether oocytes had received the xGIPC mRNA injection.
DISCUSSION

The original xGIPC-(1–320) clone (YA 5-2) isolated in the yeast two-hybrid screen was inserted in the pGAD10 vector with a reverse orientation relative to that of GAL4AD. This unusual result meant that xGIPC-(1–320) was not made as a GAL4AD fusion protein or driven by the ADH promoter. Instead it was transcribed from an intrinsic promoter lying within the ADH1 termination sequences (17) and translated as a nonfusion protein. Under this circumstance, xGIPC-(1–320) should bind the bait (GAL4DBD-xIGF-1R cyto) but should also function as a “transcription activator” capable of replacing the GAL4AD in activating the Gal1-driven reporters in the two-hybrid assay. This is not surprising given that more than 1% of random bacterial genomic fragments demonstrate transcription activator function when fused in frame with the yeast GAL4 DNA-binding domain (22). The common feature of these transcription activators is the relative abundance of acidic amino acids (22). Indeed, xGIPC-(1–320) is an acidic protein (overall pI of 6.16) with a particularly acidic C terminus (pI of 4.71 over the last 114 amino acids). A second possibility was for xGIPC-(1–320) to simultaneously bind the bait and a GAL4AD fusion protein derived from the nonsense direction of xGIPC-(1–320).

A recent study (23) indicates that the PDZ domain of malian GIPC is sufficient to mediate interaction to TrkA (receptor for nerve growth factor). Lou et al. (23) have also defined the juxtamembrane domain of TrkA as the likely docking site...
for the GIPC PDZ domain. Limited, but noticeable, sequence homology was indeed found between the juxtamembrane domains of TrkA and xIGF-1 receptor (not shown). We have not yet determined whether this region of the xIGF-1 receptor is similarly required for interacting with xGIPC. Our data also indicate that both xGIPC(1–320) and xGIPC_{N,PDZ} are capable of binding to xIGF-1 receptor, consistent with the notion that the PDZ domain is also involved in binding the intracellular domain of xIGF-1 receptor. However, the PDZ domain alone did not suffice to bind xIGF-1 receptor either in yeast two-hybrid assays (Fig. 3B) or in COS cells (not shown). Further studies will be required to clarify the nature of this apparent difference.

Clearly the most interesting implication of our data lies in the possible link between IGF-1 receptor signaling and G protein functions during oocyte maturation. High levels of cAMP are thought to be responsible for maintaining oocyte meiosis arrest (24). Progesterone, the natural maturation-inducing ovarian hormone, binds to the cytoplasmic progesterone receptor (xPR) (25, 26) and triggers a reduction of cAMP by inhibiting oocyte adenyl cyclase (27, 28). Insulin and IGF-1 can also induce maturation in vitro (29) by activating the endogenous IGF-1 receptor (7). Forskolin, a potent adenyl cyclase activator, similarly blocks both progesterone-induced and insulin-induced oocyte maturation (30, 31), suggesting that a reduction of cAMP is a necessary event in both cases. It has been reported that insulin is able to stimulate oocyte cAMP phosphodiesterase activity (32). A recent study has provided a possible link between the insulin/IGF-1 receptor and the activation of oocyte phosphodiesterase. Andersen et al. (33) reported that injection of protein kinase B/Akt results in activation of cAMP phosphodiesterase (PDE3) and induction of oocyte maturation. Protein kinase B/Akt is a serine/threonine kinase activated downstream of PI 3-kinase. We (6) and others (34) have previously shown that insulin/IGF-1 receptor activates PI 3-kinase in frog oocytes. Therefore, one signaling pathway may consist of IGF-1 receptor-activated PI 3-kinase-protein kinase B/Akt, which directly or indirectly activates oocyte PDE3 (33) and ultimately results in the reduction of oocyte cAMP.

We propose here that following ligand binding, xIGF-1 receptor activates an oocyte G_{i} in addition to the PI 3-kinase/Akt signaling pathway (32). Weifer and Weintraub (1994) (Genes Dev. 8, 1434–1447) showed that insulin/IGF-1 receptor activates PI 3-kinase, independently of G_{i}, to activate a PDE3 pathway. The co-operation of the two signaling pathways ensures the reduction of cAMP to activate a maturation-promoting signaling pathway.

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