A PDZ Domain Protein Interacts with the C-terminal Tail of the Insulin-like Growth Factor-1 Receptor but Not with the Insulin Receptor

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In this study, we report on the isolation of a PDZ domain protein, here designated as IIP-1, insulin-like growth factor-1 (IGF-1) receptor-interacting protein-1, which binds to the IGF-1 receptor, but not to the related insulin receptor, and which is involved in the regulation of cell motility. The interaction between the IGF-1 receptor and IIP-1 as well as a splice variant IIP-1/p26 was demonstrated in the yeast two-hybrid system. Using co-precipitation experiments, we confirmed the interaction in transfected cells as well as in vitro. Analysis of deletion mutants indicates that the PDZ domain of IIP-1 mediates interaction with the C-terminal tail of the IGF-1 receptor (serine-threonine-cysteine). This finding demonstrates that the C terminus of the IGF-1 receptor acts as novel PDZ domain binding site. Immunofluorescence analysis revealed an overlapping localization of IIP-1 and the IGF-1 receptor in the breast cancer cell line MCF-7. A functional connection between IIP-1 and the IGF-1 receptor is further supported by the finding that the level of expression of IIP-1 and the IGF-1 receptor strongly correlates in different normal and cancer cells. Furthermore, overexpression of IIP-1 resulted in an attenuation of migration of MCF-7 cells, which is one of the biological activities mediated by the IGF-1 signaling system.

Signaling by the insulin-like growth factor-1 (IGF-1) receptor plays a crucial role in cellular growth, migration, and survival (1). The IGF-1 receptor belongs to the family of receptor tyrosine kinases and shares a high degree of homology with the insulin receptor (2). Several signaling proteins are known to interact with the cytoplasmic part of the IGF-1 receptor, the insulin receptor and several other receptor tyrosine kinases. For example, it was shown that p85, the regulatory domain of the PI3K, interacts with the IGF-1 receptor (3, 4). However, p85 also binds to many other receptor tyrosine kinases of virtually all sub-families. Another binding partner of the IGF-1 receptor defined by two-hybrid screening is Grb 10, which binds also to other receptor tyrosine kinases like the insulin receptor and c-Ret (5–7). IRS-1, IRS-2, and SHC were also found to interact both with the IGF-1 receptor and the insulin receptor (5, 8, 9). More recently, mSH2B was described as IGF-1 receptor binding protein, which also interacts with the insulin receptor (10). 14-3-3 proteins were shown to interact with the IGF-1 receptor but not the insulin receptor (11). However, 14-3-3 proteins also influence insulin signaling via binding to IRS-1 (12). Taken together, relatively little is known about specific signal transducers of the IGF-1 receptor.

The primary physiological role of the insulin receptor is to regulate metabolic events, whereas the IGF-1 receptor activated by its ligands has a major impact on mitogenicity, transformation, and survival (13). In addition, signaling by the IGF-1 receptor is implicated in the chemotactic activity of tumor cells (14). Thus, despite the similarity of the amino acid sequence and many shared signaling proteins, unique biological effector molecules for the IGF-1 receptor and the insulin receptor are likely to participate in transducing the specific responses of these receptors. The finding that signaling of receptor chimera, in which the cytoplasmic domain of the IGF-1 receptor was fused to the extracellular domain of the insulin receptor, caused an increased mitogenic response compared with the wild type insulin receptor, supports the hypothesis of such divergent pathways (15). The C-terminal region represents the most disparate domain between the IGF-1 receptor and the insulin receptor and is particularly important for the differences in signaling events (16–20). Thus, this region might act as a prime candidate binding site for specific signaling proteins. The knowledge of such specific protein interactions discriminating the IGF-1 receptor from the insulin receptor might open novel ways for targeted therapeutic interventions.

To elucidate the differences in the signal transduction pathways of the IGF-1 receptor and the insulin receptor, it requires the isolation of proteins binding selectively to one of these receptors. In this study, we performed a yeast two-hybrid screen with the cytoplasmic domain of the IGF-1 receptor as bait to identify such proteins. Among several proteins we identified IIP-1, a PDZ domain protein, as a specific binding partner.
for the IGF-1 receptor, which does not bind to the insulin receptor or a number of other receptor tyrosine kinases tested. PDZ proteins are known to integrate several different signaling proteins in one complex at the plasma membrane (21). Therefore, IIP-1 might cluster the IGF-1 receptor in a signaling complex, resulting in the induction of the specific cellular effects of this receptor.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid System**—The cDNA coding for the cytoplasmic domain of the human IGF-1 receptor (PDZ: aa 118–229; PDZ–N: aa 150–229; PDZ–C: aa 129–188) was produced by RT-PCR using a cDNA of IIP-1, which was blocked at nucleotides 421–427 by the T7 promoter sequence. The cDNA for a GAGATAACAGCAGGCTGG3′ reverse primer with an RI/I adaptor: 5′-TCCGGGAATTCCCGGGGAGAAAGA−SalI adaptor: 5′-TCCGGAATTCCCGGGGAGAAAGA−SalI restriction sites. A RI/I restriction site at nt 1167, the insulin receptor kinase-inactive mutant (muIGF-1R: K1033A) was fused to the LexA domain, were chosen for screening a VP16-activation-domain cDNA library from E10.5 mouse embryos (22) and a Gal4-activation-domain cDNA library from Jurkat cells (23). VP16 plasmids and Gal4 plasmids from yeast strains, 2.5×10^6 HEK 293 cells were co-transfected with 5 μg of pCDNA3.1FLAG-IIP-1 or pCDNA3.1FLAG-p85β and 5 μg of a pKB5 expression vector (27) coding for the wild-type or kinase-inactive mutant (K1033A) of the IGF-1 receptor or 5 μg of a pCMV plasmid (28) encoding the insulin receptor. For transfection in HEK 293 cells the FLAG-IIP-1 was cloned in the pTre plasmid (CLONTECH). Using anti-FLAG M2 agarose (Sigma, 50 μg/vial) immunoprecipitation experiments were performed 24 h post-transfection as described previously (15), and precipitates were analyzed by immunoblotting.

**Immunofluorescence Staining of MCF-7 Cells—5×10^6 MCF-7 cells were seeded on four-well coverslips (Falcon) and transfected with 1 μg of pCDNA3.1FLAG-IIP-1 using the FuGene transfection reagent. 24 h post-transfection cells were fixed with 4% paraformaldehyde, 4% sucrose in 100 mM phosphate buffer (100 mM sodium phosphate, pH 7.4/150 mM NaCl) for 25 min. Fixed cells were blocked and permeabilized with blocking solution (16.7% FCS [v/v]/0.3% Triton X-100 [v/v] in 100 mM phosphate buffer) and then incubated with primary antibodies in blocking solution (purified anti-IGF-1 antisera 1:10, monoclonal anti-IGF-1 receptor Ab-1, Oncogene, 2 μg/ml) for 2 h, followed by a 90-min incubation with secondary antibodies (Cy2-conjugated goat anti-rabbit IgGs and Cy3-conjugated goat anti-mouse IgGs, Jackson Immunoresearch Laboratories). Some blots were stripped by incubation for 45 min at 50 °C in 62.5 mM Tris, pH 6.8, 2% SDS (w/v), and 100 μM β-mercaptoethanol and then reprobed with other antibodies as indicated in the figure legends.

**Co-immunoprecipitation Experiments—IIP-1 and p85β (GenBank accession number Y13569, aa 591–722) were co-expressed in the mammals mammalian expression constructs and the yeast strain BL21. As control, GST alone was expressed in BL21. GST fusion proteins were purified with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. IGF-1 receptor-overexpressing NIH3T3 cells (I24) were serum-starved for 24 h. 1×10^6 serum-starved cells or cells stimulated with 10 ng/ml IGF-1 (Roche Biochemicals) for 10 min were solubilized in 300 μl of lysis buffer. GST fusion proteins (5 μg) adsorbed to 50 μl of glutathione-Sepharose 4B beads (50% v/v) were added to the cell lysates and incubated overnight at 4 °C. Samples were washed three times with 1 ml ice-cold lysis buffer. The bound proteins were solubilized in SDS loading buffer and analyzed by immunoblotting.
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(Proteomics). Cell clones were isolated and maintained in DMEM containing the respective selection markers. Expression of FLAG-IIP-1 was induced with 1 μg/ml doxycycline (CLONTECH).

Cell Migration Assays—Cell migration experiments were performed with MCF-7 transfectants in Transwell chambers as described previously (29). Expression of IIP-1 was induced with 1 μg/ml doxycycline 24 h before starting the migration assay. Cells were detached with cell dissociation solution (Sigma), resuspended in serum free medium in the presence and absence of 1 μg/ml doxycycline, and seeded in the upper chamber of 12-well Transwells (12/plate) at a density of 2 × 10^5 cells/well. The lower chamber was loaded with 20% FCS in DMEM with and without 1 μg/ml doxycycline. After 72 h of incubation at 37 °C, 1/10 volume MTT (Sigma; 5 mg/ml in PBS) was added to the upper and lower chamber and incubated for 4 h at 37 °C. The resulting formazan salt crystals were scraped from the upper part of the membrane, transferred to a reaction tube, and solubilized in 500 μl of solubilization solution (100 ml of isopropanol/80 μl of 37% HCl/10 mg of SDS) overnight at 37 °C. Medium was removed from the lower chamber, and formazan crystals on the lower part of the membrane were solubilized with 500 μl of solubilization solution overnight at 37 °C. The solubilized formazan products of the upper and lower chambers were spectrophotometrically quantified at 550 nm with a reference wavelength of 690 nm using an enzyme-linked immunosorbent assay reader.

Cell Proliferation Assays—Cell proliferation experiments were also performed with MCF-7 transfectants. Cells were seeded in 96-well plates at a density of 1 × 10^4 cells/well in 1% FCS (v/v)-DMEM medium with and without 1 μg/ml doxycycline. After 24 h, cells were synchronized in 0.1% dialyzed FCS (v/v)-DMEM medium for 24 h. Cells were then cultured for 48 h in 0.1% dialyzed FCS (v/v)-DMEM medium in the presence and absence of 10^{-7} M IGF-1. Cell proliferation was quantified by WST-1 assay (Roche Biochemicals) according to the manufacturer’s instructions.

RESULTS

Identification of IIP-1 as a Specific IGF-1 Receptor-interacting Protein—To identify specific IGF-1 receptor-interacting proteins, we performed a yeast two-hybrid screen with the cytoplasmic domain of the IGF-1 receptor as bait. To achieve autophosphorylation of the bait in yeast, the cytoplasmic domain of the IGF-1 receptor was fused to the LexA DNA binding protein. This leads to dimerization and subsequent autophosphorylation of the fused bait (22) and allows isolation of proteins, which interact with the activated as well as with the quiescent IGF-1 receptor tyrosine kinase. Best phosphorylation was obtained with a LexA-IGF-1 receptor fusion protein linked via a proline-glycine spacer (data not shown). Screening of cDNA libraries with the LexA-IGF-1 receptor bait resulted in the identification of several cDNA sequences. Some of these cDNAs code for SH2 domain-containing proteins already known to bind to the IGF-1 receptor like p85PI3K, Grb10, and mSH2B (3, 6, 10). We also isolated 14-3-3β as an IGF-1 receptor binding protein. This isoform of the 14-3-3 protein family as previously (29). Expression of IIP-1 was induced with 1 μg/ml doxycycline (Roche Biochemicals). Cell clones were isolated and maintained in media containing 0.1 g/ml doxycycline. After 72 h of incubation, cells were detached with cell dissociation solution (Sigma), resuspended in serum free medium in the presence and absence of 1 μg/ml doxycycline, and seeded in the upper chamber of 12-well Transwells (12/plate) at a density of 2 × 10^5 cells/well. The lower chamber was loaded with 20% FCS in DMEM with and without 1 μg/ml doxycycline. After 24 h, cells were synchronized in 0.1% dialyzed FCS (v/v)-DMEM medium for 24 h. Cells were then cultured for 48 h in 0.1% dialyzed FCS (v/v)-DMEM medium in the presence and absence of 10^{-7} M IGF-1. Cell proliferation was quantified by WST-1 assay (Roche Biochemicals) according to the manufacturer’s instructions.

Fig. 1. IIP-1 interacts in a phosphorylation-independent manner with the IGF-1 receptor but not with several other receptor tyrosine kinases in the yeast two-hybrid system. A, interaction of proteins identified in the yeast two-hybrid screen with the IGF-1 receptor (IGF-IR), a kinase inactive mutant (muIGF-1R), and the insulin receptor (IR). IIP-1 binds to the IGF-1R and to the kinase-defective mutant, but not to the IR. APS, mSH2B, and p59fyn bind in a kinase-dependent manner to the IGF-1R and the IR. B, specific binding of IIP-1 to the IGF-1R. IIP-1 interacts with the wild type and kinase-inactive mutant of the IGF-1 receptor (IGF-1R and muIGF-1R), but not with the IR, c-Ros, c-Met, c-Kit, and c-Fms. The control p85β interacts with all of these receptor tyrosine kinases but not with the kinase inactive mutant of the IGF-1R. Colony growth assay was performed with yeast L40 cells co-transfected with the respective bait and prey cDNA constructs. Transfectants were plated on -Trp,-Leu,-His medium containing different concentrations of 3-amino-triazole (A, 0 mM; B, 20 mM). Plates were incubated for 4 days at 30 °C.
To determine whether binding of the new IGF-1 receptor interacting proteins depends upon tyrosine phosphorylation of the receptor, a kinase-inactive mutant of the IGF-1 receptor (muIGF-1R) was assessed for interaction with these proteins in the yeast two-hybrid system. IIP-1 interacted with both the quiescent and the activated receptor with similar strength (Fig. 1A). Apparently, binding of IIP-1 to the IGF-1 receptor does not depend on tyrosyl phosphorylation of the receptor. In contrast, the SH2 domain-containing proteins APS, mSH2B, p59sca, p85, and Grb10 only bound to the activated IGF-1 receptor, which is in line with the SH2-mediated phosphotyrosine interaction of these proteins. Next, we were interested to determine the binding specificity of these proteins for the IGF-1 receptor with respect to other receptor tyrosine kinases, particularly to the related insulin receptor. Therefore, we analyzed the interaction of these proteins with the cytoplasmic domain of the insulin receptor in the yeast two-hybrid system. APS, mSH2B, and p59sca interacted with the insulin receptor with similar strength as with the IGF-1 receptor (Fig. 1A). In contrast, IIP-1 did not interact with the insulin receptor despite the high homology to the IGF-1 receptor. To further prove the binding specificity of IIP-1 for the IGF-1 receptor tyrosine kinase, we tested various other receptor tyrosine kinases, for example c-Ros, c-Met, c-Ret, c-Kit, and c-Fms, for interaction with IIP-1 in the yeast two-hybrid system. Interestingly, none of these receptor tyrosine kinases showed binding to IIP-1, but all interacted with the p85 β-subunit of the PI3K (Fig. 1B). These results strongly suggest that IIP-1 is a specific binding protein of the IGF-1 receptor compared with several other receptor tyrosine kinases. Of special interest is the finding that IIP-1 does not interact with the insulin receptor, which is the closest relative of the IGF-1 receptor.

Cloning of Full-length IIP-1 and IIP-1/p26—Screening of a mouse embryo cDNA library resulted in isolation of two partial cDNA clones of IIP-1. Based on expressed sequence tag sequences, human full-length IIP-1 was assembled and cloned from MCF-7 poly(A) RNA by RT-PCR. We obtained two fragments, a longer one with 1076 nt in length, which corresponds to the cDNA of IIP-1 and a shorter one with 759 nt in length (Fig. 2A). Sequence analysis revealed that nt 145–462 of the full-length cDNA of IIP-1, including the start codon, are absent in the smaller PCR fragment. To elucidate whether the shorter IIP-1 cDNA might be due to alternative splicing, we analyzed the genomic organization of IIP1. The IIP-1 cDNA is encoded by exon 8 of IIP-1 (Fig. 2C). Interestingly, exon 3 consists exactly of the 317 nt that are absent in the smaller PCR product. This indicates that we isolated an alternative spliced form of IIP-1, which is 83 nt shorter than the full-length cDNA. Translation of IIP-1/p26 most likely starts at the first ATG in exon 4. The cDNA of IIP-1/p26 encodes aa 8–333 of IIP-1 (Fig. 2B). It consists of a part of the N-terminal region, the PDZ domain, and the C-terminal region of IIP-1 and interacts with the activated and quiescent IGF-1 receptor (Fig. 2C).

IIP-1 Binds to the IGF-1 Receptor via Its PDZ Domain—We identified two overlapping cDNA clones of IIP-1 in the two-hybrid screen, IIP-1a and IIP-1b (Fig. 3A). Both contain the PDZ domain of IIP-1 (aa 132–213) and bind to the IGF-1 receptor. IIP-1a (aa 116–323) consists of the PDZ domain and the C-terminal region of IIP-1. IIP-1b (aa 8–214) lacks the C terminus but spans over the N-terminal region and the PDZ domain. This finding already indicates that the PDZ domain plays an essential role in binding of IIP-1 to the IGF-1 receptor. To further investigate the role of the PDZ domain in the interaction with the IGF-1 receptor, we analyzed various deletion mutants of IIP-1 for interaction with the IGF-1 receptor in the yeast two-hybrid system. The PDZ domain alone (PDZ) was sufficient to mediate interaction to the IGF-1 receptor (Fig. 3A), whereas N- or C-terminal deletions into the PDZ domain (PDZΔN, PDZΔC) caused a complete loss of binding to the IGF-1 receptor. These data clearly show that interaction between IIP-1 and the IGF-1 receptor occurs via the PDZ domain of IIP-1.

IIP-1 Binds to the C Terminus of the IGF-1 Receptor—PDZ domains are known to bind to the C terminus of their interaction partners (21). Therefore, we tested a LexA bait construct coding for the C-terminal 11 amino acids of the IGF-1 receptor (IGF-1R*) for interaction with IIP-1 using yeast colony growth assays. Interaction was quantified by β-galactosidase assays. Interestingly, we found that the C-terminal 11 amino acids of
the IGF-1R* are sufficient to mediate binding to IIP-1 (Fig. 3B). Binding occurred at a similar strength compared with the entire cytoplasmic domain fused to LexA (Fig. 3B, lower panel). In contrast, IIP-1 did not bind to the C-terminal 11 amino acids of the insulin receptor (IR*) despite the fact that 5 amino acids of the insulin receptor tail are identical to the IGF-1 receptor tail. The particular importance of the most distal 3 amino acids in the C-terminal tail of the IGF-1 receptor (STC) as binding site for IIP-1 could be demonstrated by using hybrid constructs. A mutated insulin receptor tail carrying the terminal 3 amino acids of the IGF-1 receptor (IR*NPS → STC) was now able to bind to IIP-1. On the other hand, mutations of any of the terminal three amino acids in the IGF-1 receptor tail to the corresponding amino acids of the insulin receptor abolished interaction with IIP-1 (IGF-1R*′S → N, IGF-1R*′T → P, IGF-1R*′C → S). These data demonstrate that the C-terminal tip of the IGF-1 receptor serves as the docking site for the PDZ domain of IIP-1.

Interaction of Purified IIP-1 with the IGF-1 Receptor—To further examine the interaction between IIP-1 and the IGF-1 receptor, we performed an in vitro binding assay. Bacterially expressed GST-IIP-1 was bound to glutathione-Sepharose and assessed for the ability to precipitate the IGF-1 receptor from IGF-1-stimulated or unstimulated I24 cells. The IGF-1 receptor indeed bound to GST-IIP-1. Both forms of the receptor, the unphosphorylated and the phosphorylated, were precipitated with GST-IIP-1 (Fig. 4, lanes 1–2). In contrast, GST-p85β associated with the stimulated but not the quiescent IGF-1 receptor (Fig. 4, lanes 3–4). As expected, GST alone did not precipitate the IGF-1 receptor from stimulated or unstimulated I24 cells. These results demonstrate the specific interaction between purified IIP-1 and the IGF-1 receptor in vitro.

Interaction of IIP-1 with the IGF-1 Receptor in Mammalian Cells—To investigate interaction of IIP-1 with the IGF-1 receptor in mammalian cells, we transiently co-expressed FLAG-tagged IIP-1 and the IGF-1 receptor in HEK 293 cells. Protein complexes containing IIP-1 were immunoprecipitated with an anti-FLAG agarose. Also in this experimental setting IIP-1 interacts with the wtIGF-1 receptor (wt) and the kinase inactive mutant (mut) (Fig. 5A, lanes 1–2) confirming the data obtained in vitro and in yeast. As expected, p85β associates with the wtIGF-1 receptor but not with the kinase-defective receptor (Fig. 5A, lanes 3–4). To analyze the binding specificity of IIP-1 for the IGF-1 receptor with respect to the insulin receptor in mammalian cells, we co-transfected eucaryotic expression plas-
A, anti-FLAG immunoprecipitation experiments with lysates from HEK 293 cells overexpressing FLAG-tagged IIP-1 and the wt or mutant IGF-1 receptor demonstrate that IIP-1 associates with the active and quiescent IGF-1 receptor in HEK 293 cells (upper panel, lanes 1–2). FLAG-tagged p85β interacts with the phosphorylated IGF-1 receptor but not with the kinase inactive mutant (upper panel, lanes 3–4). Lysates from HEK 293 cells overexpressing the respective proteins were mixed with anti-FLAG M2 agarose. The precipitates were analyzed by immunoblotting with the anti-IGF-1Rβ antibody C-20. Expression of the IGF-1 receptor and its kinase inactive mutant in transfected HEK 293 cells was controlled by immunoblotting of lysates with the anti-IGF-1Rβ antibody C-20 (lower panel). The blot was stripped and reprobed with the anti-PY antibody 4G10 to verify phosphorylation of the wild type receptor (lower panel). Whole cell lysates also were immunoblotted with the anti-FLAG antibody M2 to confirm expression of FLAG-tagged IIP-1 and FLAG-tagged p85β in transfected HEK 293 cells (lower panel). B, IIP-1 does not associate with the insulin receptor in mammalian cells. Anti-FLAG immunoprecipitation experiments with HEK 293 cells overexpressing FLAG-tagged IIP-1 and the insulin receptor were performed as described in A. The precipitates were analyzed by immunoblotting with the anti-IR antibody C-19. FLAG-tagged IIP-1 does not bind to the insulin receptor in co-transfected HEK 293 cells (upper panel, lane 1), but FLAG-tagged p85β binds to the insulin receptor in HEK 293 cells transfected with the respective proteins (upper panel, lane 2). Expression of the insulin receptor and FLAG-tagged proteins in transfected HEK 293 cells was controlled by immunoblotting of lysates with the anti-IR antibody C-19 and the anti-FLAG antibody M2 (lower panels). The arrowhead and arrow indicate the positions of the IGF-1 receptor and insulin receptor precursor and the receptor β-subunits, respectively. The asterisk and the circle mark the position of FLAG-tagged IIP-1 and FLAG-tagged p85β, respectively. C, distribution of IIP-1 and the IGF-1 receptor in MCF-7 cells. MCF-7 cells transiently expressing IIP-1 (see arrows) were co-stained with the anti-IIP-1 antiserum and the anti-IGF-Rα antibody Ab-1 and analyzed by immunofluorescence microscopy. IIP-1 expression is dominant in the cytoplasm in close proximity to the membrane and overlaps with the membrane-associated localization of the IGF-1 receptor. Bar, 20 μm.

mids coding for FLAG-tagged IIP-1 and the wt insulin receptor in HEK 293 cells and performed immunoprecipitation assays as described above. Protein complexes consisting of the IR and p85β, but not of the IR and IIP-1, were precipitated from transfected HEK 293 cells (Fig. 5B). These experiments demonstrate that IIP-1 binds specifically and in a kinase-independent manner to the IGF-1 receptor but not to the insulin receptor in mammalian cells.

**Distribution of IIP-1 in MCF-7 Cells**—Functional interaction requires co-localization of IIP-1 with the membrane-associated IGF-1 receptor. To determine the localization of IIP-1 and the IGF-1 receptor in mammalian cells, we performed immunofluorescence assays. MCF-7 cells transiently transfected with full-length IIP-1 were co-stained with the anti-IIP-1 antiserum and the anti-IGF-1Rα antibody Ab-1. Immunofluorescence microscopy (Fig. 5C) revealed that the IGF-1 receptor is located at the cell membrane. Expression of IIP-1 was preferentially detected in the cytoplasm in close proximity to the membrane. Weak staining of endogenous IIP-1 is seen in untransfected cells, whereas transfected cells show a strong staining for recombinant IIP-1 (see arrows). The results of the immunofluorescence studies suggest that IIP-1 physically associates with the membrane-spanning IGF-1 receptor in mammalian cells.

**Expression of IIP-1 and the IGF-1 Receptor Correlates in Different Cancer Cell Lines and Normal Cells**—Next we investigated various human cancer cell lines and normal cells for their expression of IIP-1 and the IGF-1 receptor. Western blot analysis revealed that IIP-1 as well as the IGF-1 receptor are expressed in many adherent and non-adherent cells (Fig. 6). The expression pattern of IIP-1 and the IGF-1 receptor correlates in the various cell types. Cell lines and normal cells showing high expression of IIP-1 are the melanoma cell line G361, the adenocarcinoma cell lines SW480 and MCF-7 (lanes 1–3), normal breast epithelial cells (human mammary epithelial cells), small airway epithelial cells (lanes 7–8), and keratinocytes (lane 11). Interestingly, expression of the IGF-1 receptor is also high in all of these cell lines. In contrast, the T-cell leukemia cell line MOLT-3 and the Burkitt lymphoma cell line Daudi (lanes 4–5) as well as endothelial human umbilical vascular endothelial cells and human fibroblasts (lanes 9–10) show low expression of IIP-1 and the IGF-1 receptor. Moreover, expression of IIP-1 and the IGF-1 receptor is very low in the promyelocytic leukemia cell line HL-60 (lane 6) and in peripheral blood lymphocytes (lane 12). These data implicate that IIP-1 and the IGF-1 receptor might be tightly co-expressed in many different cell types suggesting a functional connection between both proteins. In general, cells of epithelial origin show a higher expression of both IIP-1 and IGF-1 receptor than other cell types.

**IIP-1 Inhibits Migration of MCF-7 Cells**—Signaling by the IGF-1 receptor tyrosine kinase mediates several cellular func-
motility compared with the non-induced transfectants. Thus, aberrant expression of IIP-1 interferes with the migration of the breast cancer cell line MCF-7, whereas cell proliferation and survival are not affected.

**DISCUSSION**

The IGF-1 receptor shares a number of signal transducers and pathways with other receptor tyrosine kinases. However, more specific signaling proteins might also exist, which are responsible for particular cellular functions induced by the IGFs. We performed a yeast two-hybrid screen to identify such IGF-1 receptor interacting proteins and isolated several cDNAs coding for SH2 domain signaling proteins such as APS and p59γ, which have not yet been described as binders for the IGF-1 receptor. However, APS and p59γ bind to the insulin receptor and to other receptor tyrosine kinases as well (30, 37-42).

Interestingly, we also isolated a PDZ domain-containing protein, designated as IIP-1, which is a new binder of the IGF-1 receptor. A phosphorylation-independent interaction of IIP-1 and the IGF-1 receptor was detected in vitro as well as in yeast and in mammalian cells. Moreover, a splice variant, IIP-1/p26, was identified, which interacts both with the activated and quiescent IGF-1 receptor. IIP-1/p26 consists of a truncated N-terminal region, the central PDZ domain, and the C-terminal region of IIP-1.

Here, we characterized the PDZ domain of IIP-1 as the docking site for the IGF-1 receptor. PDZ domain proteins are often localized to highly specialized submembranous sites, suggesting their participation in cellular junction formation, receptor or channel clustering, and intracellular signaling events (21). Many PDZ proteins act as docking sites for different binding partners. In addition to the IGF-1 receptor, other binding partners for the PDZ domain of IIP-1 have also been described. IIP-1 was identified as GIPC, a protein interacting with the RGS protein GAIP (34), as Semcap-1, which binds to the semaphorin M-Sem-F (35), as Npn-1 interacting protein NIP (43) and as Synectin, a Syndecan-4 binding protein (36). Thus, IIP-1 might provide a link between the IGF-1 receptor system and other signaling pathways.

PDZ domains are found in a broad variety of proteins with diverse functions, for example in membrane-associated guanylate kinases, cytoskeletal proteins, protein tyrosine phosphatases, and serine/threonine kinases (21). An emergent class of interaction partners for PDZ domain proteins are receptor tyrosine kinases. Several PDZ proteins mediate the correct localization of members of the EGF receptor tyrosine kinase family to distinct membrane regions. The PDZ protein PSD-95 is involved in localizing ErbB4 to the postsynaptic density structure (44) and Erbin plays a role in the localization of ErbB2 to basolateral membrane structures (45). In Caenorhabditis elegans, the EGF receptor homologue let-23 forms a complex with the PDZ proteins lin-2, lin-7, and lin-10 at basolateral sites of vulval epithelial cells (46). Other receptor tyrosine kinases are also binding partners for PDZ proteins. Several Eph receptors interact with the ras binding protein AF-6 (47, 48), the PDGF receptor associates with the Na+/H+ exchanger regulatory factor that potentiates receptor activity (49), and c-Kit interacts with the multiple PDZ domain-containing protein MUPP-1 (50).

In this study, we describe IIP-1, a PDZ domain-containing protein specifically binding to the IGF-1 receptor tyrosine kinase. IIP-1 does not bind to the highly related insulin receptor neither in the yeast two-hybrid system nor in mammalian cells. Also, no association of IIP-1 with the receptor tyrosine kinases c-Ros, c-Met, c-Ret, c-Kit, and c-Fms was observed. Thus, IIP-1 is a remarkably specific binding protein for the IGF-1 receptor.
with respect to other receptor tyrosine kinases. Apart from 14-3-3 proteins (11), IIP-1 is the only known protein interacting with the IGF-1 receptor but not the insulin receptor. Therefore, IIP-1 and 14-3-3 might participate in mediating some of the specific cellular effects of the IGF-1 receptor as survival and motility, which are distinct from the metabolic effects mediated by the insulin receptor.

We found that binding of IIP-1 to the IGF-1 receptor is mediated by the C-terminal tail of the receptor whereby each of the last three amino acids is important for interaction with IIP-1. Replacement of these amino acids with the corresponding amino acids of the insulin receptor resulted in a complete loss of interaction. These results are in line with the finding that most PDZ proteins interact with the extreme C terminus of their binding partners (21). Only few examples exist for internal binding sites for PDZ domains (51). Two different C-terminal consensus-binding sites of PDZ proteins are known. Type I PDZ proteins bind to the consensus (S/T)X(V/I), type II PDZ proteins recognize the consensus (F/Y/X/A/P/V) (21, 52). The binding sites in the IGF-1 receptor (STC), GAIP (SEA), Npn-1 (SEA), and in M-SemF (SSV) perfectly match or resemble type I motifs, whereas the C terminus of Syndecan-4 (FYA) represents a type II motif. Thus, binding of IIP-1 to its interaction partners is mediated by bona fide type I and type II and by other binding motifs like STC. The other receptor tyrosine kinases tested that did not interact with IIP-1 do not contain a C terminus with an amino acid sequence related to one of the C-terminal binding sequences of IIP-1.

When this manuscript was being prepared, an interaction between IIP-1 (GIPC) and TrkA was described (53). In contrast to the interaction of IIP-1 (GIPC) and the IGF-1 receptor, which is mediated by the C terminus of the IGF-1 receptor, the interaction of IIP-1 and TrkA is mediated by the juxtamembrane region of TrkA. Lou et al. (53) found that amino acids 472–493 of TrkA are required for interaction with IIP-1 (GIPC). However, an exact binding motif was not defined. They further demonstrated that IIP-1 (GIPC) restrains TrkA in a complex with GAIP, thereby coupling the NGF receptor to G-protein signaling pathways. IIP-1 might link the IGF-1 receptor to G-protein signaling cascades in a similar way.

Our data indicate a functional connection between IIP-1 and the IGF-1 receptor. Both proteins are co-localized in mammalian cells, and the co-expression in several cancer cell lines and normal cells suggests a co-regulation of the expression of both proteins. Moreover, we have shown that overexpression of IIP-1 negatively affects the motility of MCF-7 breast carcinoma cells. These data are in line with the results of Gao et al. (54) who demonstrated that overexpression of Synectin (IIP-1) in ECV304 endothelial cells leads to a dose-dependent inhibition of migration. IGF-1 is known as a chemoattractant for several cancer and normal cells and stimulates migration of these cells (54–57). The IGF-1 receptor was also identified as a positive regulator of the invasive/metastatic phenotype (14, 58). Our data indicate that IIP-1 might modulate tumor cell invasion and metastasis.

Interestingly, IIP-1 (GIPC) also negatively affects TrkA function. Overexpression of IIP-1 (GIPC) results in a decreased activation of mitogen-activated protein (MAP) kinase after nerve growth factor stimulation (53), which is in line with our observations that IGF-1-mediated MAP kinase activation is also reduced following overexpression of IIP-1 (data not shown). However, attenuation of signals does not seem to be a general consequence of binding of PDZ proteins to receptor tyrosine kinases. Binding of PSD-95 to ErbB4 potentiates the response to its ligand neuregulin (44), and Na+/H+ exchanger regulatory factor also stimulates kinase activity of the PDGF receptor (49).

Several domains of the IGF-1 receptor have been identified, which are responsible for mediating distinct cellular function. For example, the region between amino acids 1245 and 1310 mediates the transforming activity of the receptor (17). Mutations in the C terminus at tyrosine 1251 or at histidine 1293 and lysine 1294 abolish anti-apoptotic function of the receptor (59). Here we provide evidence that the C-terminal region is involved in cell migration.

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