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Multimerization of the Protein-tyrosine Phosphatase (PTP)-like Insulin-dependent Diabetes Mellitus Autoantigens IA-2 and IA-2 β with Receptor PTPs (RPTPs)

INHIBITION OF RPTP α ENZYMATIC ACTIVITY*

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Most receptor-type protein-tyrosine phosphatases (RPTPs) contain two tandem PTP domains. For some RPTPs the enzymatically inactive membrane-distal phosphatase domains (D2) were found to bind enzymatically active membrane proximal PTP (D1) domains, and oligomerization has been proposed as a general regulatory mechanism. The RPTP-like proteins IA-2 and IA-2 β , major autoantigens in insulin-dependent diabetes mellitus, contain just a single enzymatically inactive PTP-like domain. Their physiological role is as yet enigmatic. To investigate whether the catalytically inactive cytoplasmic domains of IA-2 and IA-2 β are involved in oligomerization, we exploited interaction trap assay in yeast and glutathione S-transferase pull-down and coimmunoprecipitation strategies on lysates of transfected COS-1 cells. The results show that IA-2 and IA-2 β are capable of homo- and heterodimerization to which both the juxtamembrane region and the phosphatase-like segment can contribute. Furthermore, they can form heterodimers with some other RPTP members, most notably RPTP α and RPTP ϵ , and down-regulate RPTP α enzymatic activity. Thus, in addition to homodimerization, the enzymatic activity of receptor-type PTPs can be regulated through heterodimerization with other RPTPs, including the catalytically inactive IA-2 and IA-2 β .

ily of PTPs can be classified into cytosolic and transmembrane or receptor-like PTPs (RPTPs). The latter consist of an ectodomain, which displays the use of a bewildering variety of protein modules, a single membrane-spanning region, and one (such as in PTPBR7 and DEP1 (1–3)) but in most cases two highly conserved intracellular tyrosine-specific phosphatase domains (4, 5). Intriguingly, in RPTPs that possess two phosphatase domains the membrane-proximal (D1) domain is catalytically active, whereas the membrane-distal (D2) domain shows no or only limited activity (6). Still, D2 domains are highly conserved among species, sometimes even more so than the D1 domains (7), pointing toward significant functional relevance. Evidence is accumulating that D2 domains are involved in protein interactions that regulate RPTP activity, including homo- and heterodimerization through interactions with D1 domains and/or the so-called wedge in the membrane proximal segment (8–16). Furthermore, D2 domain-mediated interactions themselves may be regulated as witnessed for the oxidative stress-induced stabilization of RPTP α dimers (17).

RPTP-like proteins exist that have a single but inactive PTP domain, and it has been suggested that the inactive domain is used as a phosphotyrosine recognition/binding moiety (18). Two such proteins, IA-2 and IA-2 β , have drawn quite some attention because they were identified as the precursors of 40- and 37-kDa insulin-dependent diabetes mellitus-specific major autoantigens, respectively (19). IA-2 (20) (also termed PTP35 (21) or ICA512 (22)) and IA-2 β (23) (also known as Phogrin (24), PTP-NP (25), ICAAR (26), or IAR (27)) are membrane-spanning RPTP-like proteins, with a cysteine-rich region at their N-terminal extracellular part, a single-pass transmembrane region, and a single inactive intracellular PTP domain. The lack of phosphotyrosine-specific activity is due to some major substitutions of conserved amino acids within the PTP domain that are critical for the specific activity of PTPs against substrates. Specifically, the conserved proline in the so-called WPD loop is changed into a tyrosine, and the obligatory alanine close to the catalytic cysteine is an aspartic acid residue in the IA2 sequence. In IA-2 β the catalytic aspartate of conventional PTPs in the WPD loop is replaced by an alanine, and again an aspartate replaces the obligatory alanine at position +2 from the catalytic cysteine of active PTPs. Indeed, appropriate back-mutations rendered the IA-2 PTP-like molecule catalytically active (28), in line with similar findings for inactive D2 PTP domains (29–31).

Many investigations are focused on the role of IA-2 and IA-2 β in autoimmune diabetes, but only limited information is avail-

One of the major regulatory mechanisms in signal transduction pathways is the phosphorylation on tyrosine residues. Two enzyme classes exert the delicate control of phosphotyrosine levels within cells: protein-tyrosine kinases (PTKs)¹ and protein-tyrosine phosphatases (PTPs). Members of the superfam-

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¹ The abbreviations used are: PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; RPTP, receptor PTP; GST, glutathione S-transferase; GFP, green fluorescent protein; ERK, extracellular signal-regulated kinase; JM, juxtamembrane; PD, phosphatase-like domain; PDZ, PSD-95, DlgA, and 20-1; VSV, vesicular stomatitis virus.

able about the physiological role of these RPTP-like proteins. Their expression pattern is restricted to cells of neuroendocrine origin where they are found as intrinsic membrane proteins of secretory granules (24, 32). Several studies have suggested their association with the cell cytoskeleton (32–34), which may be increased upon stimulation of insulin secretion (33). Recently, the binding of IA-2 to the PDZ domain of β 2-syntrophin and neuronal nitric-oxide synthase in pancreatic beta cells has been reported (35), and a model showing how IA-2 can link secretory granules with the cytoskeleton and signaling pathways regulating secretion has been proposed (36). Both IA-2 and IA-2 β also bind to the carboxyl-terminal domain of a novel spectrin, β IV spectrin, and, although the intracellular localization is different, a transient interaction *in vivo* during the intracellular route of this protein cannot be excluded (37). This suggests that perhaps IA-2 and IA-2 β are involved in the regulation of neuropeptide or peptide hormone secretory pathways, and some data even imply reversible phosphorylation of these RPTP-like proteins in the regulation of secretory-granule mobilization and recruitment in exocytosis (38).

Here we report that IA-2 and IA-2 β form homo- and heterodimers and can associate with other RPTP members. Importantly, co-expression of IA-2 and IA-2 β together with RPTP α reduces the enzymatic activity of the latter. Oligomerization has been proposed as a regulatory mechanism for catalytically active PTPs (15). Our findings indicate that the regulation of the enzymatic activity of RPTPs may in addition depend on the association with RPTP-like, catalytically inactive family members such as IA-2 and IA-2 β .

MATERIALS AND METHODS

Plasmids and Constructs—PCR fragments encompassing different regions of IA2 and IA-2 β were cloned in pEG202 or pJG4-5 for the two-hybrid binding assay to create a fusion protein with the LexA DNA-binding domain, as well as a fusion with the LexA DNA activation domain, respectively, in pGEX to make GST fusion proteins, in pSG8VSV to make VSV-tagged fusion proteins or in pEGFP to obtain GFP fusion proteins. The different domains of IA-2 and IA-2 β encompassed the following residues: the cytoplasmic domain of IA-2 (amino acid residues 601–979, GenBankTM accession number Q60673), the phosphatase domain of IA-2 (amino acids 733–979), the juxtamembrane region of IA-2 (amino acids 601–732), the cytoplasmic domain of IA-2 β (amino residues 622–1001, accession number P80560), the IA-2 β phosphatase domain (amino acids 767–1001), and the juxtamembrane region of IA-2 β (residues 636–785). To generate expression plasmids for carboxyl-terminally myc epitope-tagged IA-2 and IA-2 β , the stop codon was replaced by an oligonucleotide sequence encoding a *Bam*HI or *Bgl*II site, respectively, using PCR. Restriction fragments of the phosphatase cDNAs were ligated into a cytomegalovirus promoter-based expression vector in a way that the open reading frame of the cDNAs was fused in-frame to a sequence encoding a five times-repeated myc epitope in the vector. All PCR-generated constructs were checked for mutations by automated DNA sequencing.

The two-hybrid plasmid containing the phosphatase domain of PTP-BL and plasmids expressing VSV-tagged RPTPs DEP-1, RPTP σ , and RPTP α or myc-tagged domains of RPTP ϵ , RPTP μ , and RPTP α have been described previously (14, 39, 40). The yeast two-hybrid plasmid containing the segment encoding 409 carboxyl-terminal residues of PTP-SL (which include the PTP domain) and the mammalian expression plasmids pSG8/PTP-SLcyt-VSV and pMyc-ERK2 will be described elsewhere.

Two-hybrid Assay—Plasmid DNAs and the yeast strain used for the interaction trap assay were provided by Dr. Roger Brent and colleagues (Massachusetts General Hospital, Boston, MA) and used as described previously (41). Plasmids expressing the cytoplasmic domains of IA-2 or IA-2 β fused to the LexA transcriptional activation or DNA-binding domain were introduced in yeast strain EGY48 (*MATa trp1 ura3 his3 LEU2::pLexAop6-LEU2*) containing the reporter plasmid pSH18-34, which includes the *lacZ* reporter gene. Interactions were validated by growth and blue coloring on minimal agar plates lacking histidine, tryptophan, uracil, and leucine, which contain 2% galactose, 1% raffinose, and 80 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, buffered at pH 7.0.

Cells, Transfections, and Antibodies—COS1 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal calf serum. Transient transfection of COS-1 cells was performed by the DEAE-dextran method. In brief, for a 10-cm dish 9-ml serum-free medium was mixed with 15 μ l of chloroquine (100 mM), 450 μ l of DEAE-dextran (1 mg/ml), and 5 μ g of plasmid DNA. Cells were washed once with serum-free medium, and the pre-mixed solution was added to the cells. Following a 2.5-h incubation, cells were shocked with 10% Me₂SO in phosphate-buffered saline for 2 min. After gentle replacement of the Me₂SO-containing medium, the cells were cultured for 24 h in Dulbecco's modified Eagle's medium containing 5% fetal calf serum.

Monoclonal antibodies P5D4 (reactive against the VSV-G epitope-tag) and 9E10 (against the myc epitope tag) as well as the GFP-reactive polyclonal antiserum have been described previously (42–44). Polyvalent rabbit antiserum against IA-2 was a kind gift of Dr. Michael Christie (London, UK). Monoclonal antibody against GFP and polyvalent antiserum against the Myc tag were from Santa Cruz Biotechnology (Santa Cruz, CA).

GST Pull-down Assay—The cDNA parts encoding the cytoplasmic domains of IA-2 and IA-2 β were amplified by PCR and subcloned into the pGEX vector for isopropyl-1-thio- β -D-galactopyranoside-induced expression in bacterial host *Escherichia coli* BL21(DE3). The recombinant proteins were purified with glutathione 4B-Sepharose beads as described previously (45). Triton X-100-soluble extracts (in buffer A: 50 mM HEPES, pH 7.5; 150 mM NaCl; 1.5 mM MgCl₂; 1 mM EGTA; 10% glycerol; 1% Triton X-100; 1 mM phenylmethylsulfonyl fluoride; 10 μ g/ml aprotinin; 10 μ g/ml leupeptin; 10 μ g/ml pepstatin A) of transfected COS1 cells were incubated overnight at 4 °C with unloaded glutathione 4B-Sepharose beads or with beads loaded with 2 μ g of GST, GST-IA2cyt, or GST-IA2 β cyt, respectively. Beads were then washed five times with buffer A, and bound proteins were eluted in SDS sample buffer, subjected to SDS-PAGE, and analyzed by Western blotting with anti-VSV antibodies.

Immunoprecipitation—Cells were harvested and lysed by scraping in 500 μ l of ice-cold buffer A. After 5 min of incubation on ice, the lysate was centrifuged for 20 min at 13,000 $\times g$ at 4 °C. Cleared lysates were used for immunoprecipitation with 5 μ l of protein A-Sepharose CL-4B or protein G-Sepharose 4 Fast Flow beads preloaded with monoclonal antibody P5D4 (2 μ l of ascites fluid) or 9E10 (1 ml of hybridoma culture supernatant) at 4 °C overnight, while rotating. The immunoprecipitates were washed four times with 1.0 ml of lysis buffer and boiled for 5 min in 20 μ l of 2 \times sample buffer (100 mM Tris-HCl, pH 6.8; 200 mM dithiothreitol; 4% SDS; 0.2% bromophenol blue; 20% glycerol). Proteins were resolved on 12% polyacrylamide gels and transferred to nitrocellulose membranes by Western blotting. Blots were blocked using 5% nonfat dry milk in TBST (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1% Tween 20). Incubations with primary antibodies were done overnight at 4 °C. Incubations with secondary antibodies (20,000 times diluted peroxidase-conjugated goat anti-mouse or rabbit IgG), and subsequent washes were done in TBST at room temperature. Labeled bands were visualized using freshly prepared chemiluminescent substrate (100 mM Tris-HCl, pH 8.5; 1.25 mM *p*-coumaric acid; 0.2 mM Luminol; 0.009% H₂O₂).

PTP Assay—COS1 cells, seeded in 6-cm dishes, were transiently transfected as indicated above. Cell extracts were obtained in the absence of phosphatase inhibitors, and assayed for PTP activity using pNPP as a substrate. Before harvesting, cells were washed twice with ice-cold phosphate-buffered saline. 200 μ l of PTP assay buffer (0.1 mM succinic acid, pH 6.9; 150 mM NaCl; 1 mM EDTA; note the absence of phosphatase inhibitors) was added and cells were scraped. After freezing in liquid nitrogen and thawing, the cells were sonicated twice for 10 s. The obtained lysates were cleared by centrifugation at 20,000 $\times g$ at 4 °C for 20 min, and equal amounts of the extracts were analyzed directly by SDS-PAGE and immunoblotting. Obtained immunoreactivity for RPTP α protein was quantified by densitometric scanning of the ECL-generated x-ray film exposure using only the linear density range of the film. In parallel, aliquots were incubated in 500 μ l of PTP assay buffer containing 10 mM pNPP as a substrate. At two different time points (30 and 60 min) 200- μ l aliquots were taken, and the reaction was stopped by adding 200 μ l of 2 M NaOH. The A_{415 nm} value was measured spectrophotometrically.

RESULTS

The Cytoplasmic Domains of IA-2 and IA-2 β Interact—The recent finding of catalytically inactive membrane-distal RPTP phosphatase domain (D2) binding to enzymatically active

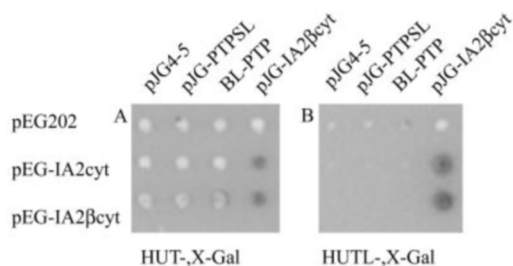


FIG. 1. Binding of the cytoplasmic domain of IA-2 β to the cytoplasmic regions of IA-2 and IA-2 β in the yeast two-hybrid system. The cDNA part encoding the cytoplasmic domain of IA-2 β was fused to the LexA transactivation domain in the pJG4-5 vector and transfected in the EGY48 yeast strain expressing the LexA DNA-binding domain fusions of the IA-2 or IA-2 β cytoplasmic domain. The bait-prey combinations were scored for interaction potential using a β -galactosidase activity-dependent blue coloring assay alone (A) or in combination with growth selection on medium lacking leucine (B). Only IA-2 and/or IA-2 β combinations scored positively. The cytosolic part of PTPBR7, identical to the last 409 residues of PTP-SL (3) and the carboxyl-terminal 360 amino acids of PTP-BL (40), representing PTP domains of unrelated phosphatases, did not interact with IA-2 or IA-2 β , demonstrating specificity. Empty vectors were included as negative controls.

membrane proximal PTP (D1) domains firmly established the dimerization potential of PTP domains (14, 46, 47). To investigate whether the catalytically inactive cytoplasmic regions of the RPTP-like proteins IA-2 and IA-2 β , which share a high sequence homology (72%), are capable of forming dimers, we exploited the LexA-based interaction trap in yeast (41). The complete cytoplasmic portions of mouse IA-2 and IA-2 β were subcloned in the appropriate bait as well as prey vectors, and following yeast transformation the combinations were scored for binding. The results reveal both homo- and heterodimeric interactions among the cytoplasmic domains of IA-2 and IA-2 β (Fig. 1). These findings are specific, because both protein segments did not display binding to the protein encoded by the empty vector nor with phosphatase domain-containing segments of the unrelated PTP-SL/PTPBR7 (3) and PTP-BL (40).

As an independent means to underscore the observed interactions, we performed GST pull-down experiments (data not shown). Indeed, the bacterially expressed cytoplasmic region of IA-2 fused to GST (GST-IA2cyt), but not GST alone, could pull down the GFP-tagged cytoplasmic domain of IA-2 (GFP-IA2cyt) from lysates of transfected COS-1 cells. Likewise, GST-IA2 β cyt was incubated with lysates from COS-1 cells overexpressing GFP-IA2 β cyt, and again the GST fusion protein demonstrated association with GFP-IA2 β cyt. In this assay we also tested whether the intracellular domains of IA-2 and IA-2 β are capable of forming heterodimers. Indeed, the bacterially expressed cytoplasmic domain of IA-2 successfully brought down the GFP-tagged cytoplasmic domain of IA-2 β and, reciprocally, GFP-IA2cyt was pulled down by GST-IA2 β cyt (data not shown).

Finally, to confirm the observed interaction also *in vivo* in mammalian cell systems, we co-expressed VSV-tagged cytoplasmic domains of IA-2 or IA-2 β together with GFP-IA2cyt or GFP-IA2 β cyt in COS-1 cells. In all combinations GFP-IA2cyt as well as GFP-IA2 β cyt co-precipitated with VSV-IA2cyt or VSV-IA2 β cyt (Fig. 2). The amount of GFP-tagged protein co-precipitated by VSV-IA2cyt appears to be more than that in the VSV-IA2 β cyt precipitate, but this may relate to the lower expression level of IA-2 β -derived proteins. In the reverse experiment, we were also able to show that the VSV-tagged domains co-precipitate with the GFP-containing constructs (data not shown).

Heterodimers of Full-length IA-2 and IA-2 β —The above studies all depended on the use of truncated epitope-tagged

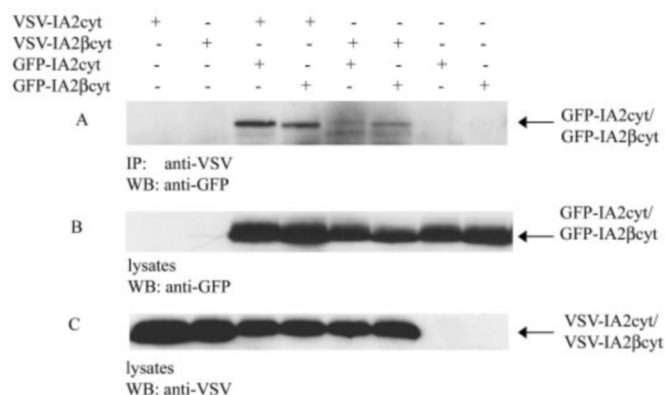


FIG. 2. The cytoplasmic regions of IA-2 and IA-2 β can be co-precipitated. COS1 cells were transiently transfected with VSV- or GFP-tagged cytoplasmic domains of IA-2 and IA-2 β in various combinations. Upon immunoprecipitation of VSV-tagged proteins from cell lysates, co-precipitating proteins were detected using anti-GFP antiserum (A). Interaction strengths for the various IA-2 and IA-2 β combinations appear comparable when taking into account their respective expression levels in the lysates (B and C).

fragments of IA-2 and IA-2 β . To investigate whether these interactions may take place under more physiological conditions, we used full-length proteins in co-immunoprecipitation experiments. In the absence of a mono-specific IA-2 β antibody, we used full-length IA-2 β that was tagged carboxyl-terminally with the VSV-G epitope in combination with full-length IA-2. The latter could be detected on Western blots using a rabbit polyclonal antiserum against IA-2 that hardly cross-reacts with IA-2 β . Upon immunoprecipitation of IA-2 β VSV indeed full-length IA-2 was only detectable with the IA-2 antiserum when both proteins were co-expressed (data not shown), demonstrating the occurrence of full-length IA-2-IA-2 β heterodimers.

To illustrate the specificity of the interaction, we performed a similar experiment but using carboxyl-terminally myc-tagged IA-2 β and VSV-tagged full-length IA-2 in combination with a VSV-tagged version of the active tyrosine phosphatase domain of PTP-SL (3) or with myc-tagged ERK2, a protein kinase. Independent of whether the myc- or the VSV-tagged protein was immunoprecipitated, we could only observe precipitation of IA-2-IA-2 β complexes (Fig. 3). No interaction of the PTP-like molecules with ERK2 protein kinase or PTP-SL tyrosine phosphatase (see also Fig. 1) was observed. Importantly, comparison of signal intensities indicated that heteromeric complex formation by the phosphatase-like molecules was extensive.

Regions Involved in the Interactions—To examine how the intracellular domains of IA-2 and IA-2 β interact, we made deletion mutants consisting of the juxtamembrane (JM) region or the phosphatase-like domain (PD) only. These mutants, all containing the VSV tag at their amino terminus, were investigated for their ability to co-immunoprecipitate with the cytoplasmic parts of IA-2 and IA-2 β (Fig. 4). As compared with the entire IA-2 cytoplasmic domain, the phosphatase domain by itself displayed a higher binding affinity toward GFP-IA2cyt and GFP-IA2 β cyt. The JM region, however, turned out to be the most effective in co-precipitating GFP-IA2cyt and GFP-IA2 β cyt. To appreciate the difference in binding we should add that the ECL exposure time used in this experiment was much shorter than that used for Fig. 2. In contrast to the single band that is visible in lysates upon direct Western blot detection with anti-GFP antiserum, two sharp bands that are ~4 kDa apart were detected following the immunoprecipitation. The lower band probably represents a degradation product rendered during the overnight precipitation.

Interactions of IA-2 and IA-2 β with Other Members of the PTP Family—Because dimerization of phosphatases has re-

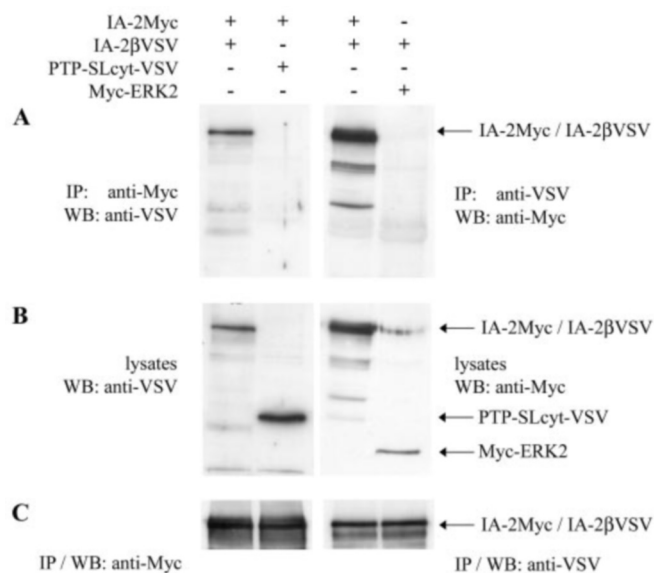


FIG. 3. Strong and specific interaction between full-length IA-2 and IA-2 β . COS1 cells were transiently transfected with constructs encoding myc-tagged full-length IA-2 or ERK2 and VSV-tagged full-length IA-2 β or PTP-SL phosphatase domain. For the *left lanes* IA-2Myc protein was immunoprecipitated from cell lysates, and coprecipitating proteins were detected using a polyvalent anti-VSV antiserum. Only IA-2 β VSV and not PTP-SLcyt-VSV was found to associate with IA-2Myc (A). The *right lanes* show results for immunoprecipitating IA-2 β VSV and subsequent detection of associating proteins using polyvalent anti-myc. IA-2Myc but not Myc-ERK2 could be observed in the precipitate (A). Expression levels of tagged proteins in the lysates (B; one-twentieth of input) and the levels of immunoprecipitated proteins (C) are displayed in the *bottom panels*. The *upper band* in the *right lane* of B results from some spill-over from the sample on the *left*.

cently been described as a regulatory mechanism for several RPTPs (9–14), we tested whether IA-2 and IA-2 β can also interact with other RPTPs. Upon incubation of bacterially produced GST-IA2cyt and GST-IA2 β cyt with lysates of COS-1 cells transfected with VSV-tagged RPTP α , RPTP σ , and DEP-1, only the non-glycosylated form of RPTP α , but not the other RPTPs, was found to bind to the GST fusion proteins (Fig. 5). The three RPTPs used in this assay all represent a different subfamily of RPTPs (5). The phosphatase domain of the protein-tyrosine phosphatases PTP-SL/PTPBR7 and PTP-BL had failed to interact with IA-2 and IA-2 β cytoplasmic domains in earlier experiments (Figs. 1 and 3), underscoring interaction specificity.

We subsequently performed co-precipitation experiments using transfected cell lysates containing myc-tagged portions of RPTP α , RPTP ϵ , and RPTP μ together with VSV-tagged cytoplasmic domains of IA-2 or IA-2 β . RPTP α and RPTP ϵ belong to the same type IV subfamily of RPTPs, characterized by their short glycosylated extracellular parts, whereas RPTP μ represents a member of the type II, cell adhesion molecule-like subfamily (4, 5). All cytoplasmic domains co-precipitated with those of IA-2 and IA-2 β . Interactions of RPTP α , RPTP ϵ , and RPTP μ cytoplasmic domains with IA-2 were more pronounced than with IA-2 β , again probably reflecting the lower expression levels of IA-2 β (data not shown).

Multiple Interaction Sites in IA-2—We used again the VSV-tagged deletion constructs harboring the phosphatase domain or juxtamembrane region separately, to determine the parts involved in the interaction with the other RPTPs. These IA2 constructs were co-expressed together with different domains of RPTP α , RPTP ϵ , and RPTP μ . All VSV-tagged proteins (VSV-IA2JM, VSV-IA2PD, and VSV-IA2cyt) were capable of interacting with single D1 or D2 phosphatase domains of RPTP α

(Fig. 6) and RPTP ϵ as well as with the D2 domain of RPTP μ (Fig. 7). Signal intensities suggest that association with the D2 domains is somewhat stronger than with the D1 domains. On the contrary, in the case that both PTP domains together are present (myc- ϵ D12 construct) the interaction with IA-2 parts is almost abolished. Again the juxtamembrane region of IA-2 is the strongest binding partner of the different domains, whereas the phosphatase domain alone shows only a weak interaction. With IA-2 β -derived constructs, essentially the same results were obtained (data not shown).

Heterodimerization with Active RPTP α Inhibits Phosphatase Activity—To investigate whether dimerization of IA-2 and IA-2 β with other RPTPs may affect their activity we overexpressed RPTP α together with epitope-tagged cytosolic and full-length IA-2 or IA-2 β proteins. We assumed that overexpressed RPTP α would contribute considerably to the overall tyrosine phosphatase activity in the lysates. Indeed, PTP activity, as measured spectrophotometrically using pNPP as an artificial substrate and normalized for the relative protein content, in extracts of RPTP α -transfected cells was some 50% higher than in control extracts (Fig. 8, *lanes 1* and *2*). Upon co-expressing tagged forms of an unrelated protein, centaurin- γ 2, as a negative control together with RPTP α no significant reduction in enzymatic activity was observed (Fig. 8, *lanes 7, 11, and 15*). On the contrary, all IA-2 and IA-2 β protein versions significantly inhibited the PTP activity due to RPTP α (Fig. 8, *lanes 3, 5, 9, and 13*) without having detectable effects on the basal PTP activity in lysates expressing the IA-2 or IA-2 β proteins alone (*lanes 4, 6, 10, and 14*). Reminiscent of the homodimerization results (see Figs. 2 and 3), the full-length IA-2 and IA-2 β proteins appeared more efficient than their cytosolic counterparts (Fig. 8, *lanes 9 and 13 versus lanes 3 and 5*). The observed inhibitory effect gains in momentum when bearing in mind that these RPTP-like proteins to a large extent will homodimerize and that thus only a fraction will be engaged in heterodimerization with RPTP α .

DISCUSSION

In this study we have shown that IA-2 and IA-2 β , two receptor-type protein-tyrosine phosphatase-like proteins that form a separate subclass within the PTP superfamily, are capable of homo- and heterodimerization. In addition, they can form heterodimers with members of other subclasses of RPTPs, most notably the type IV (RPTP α and RPTP ϵ), thereby regulating their enzymatic activity. These interactions are specific, because several other RPTPs, including PTPBR7 and DEP1, were found not to associate with IA-2 and IA-2 β cytoplasmic parts. The data also suggest that there are multiple sites of interaction, and the ones mediated by the IA-2 or IA-2 β JM region appear stronger than those exerted by the PD.

Although IA-2 and IA-2 β share a high homology with other members of the family of PTPs within their cytoplasmic domain, they are unique and clearly distinct in several ways. First of all, their discovery as being major autoantigens in insulin-dependent diabetes mellitus makes them rather unique within the family. Also their extracellular moiety, containing potential glycosylation sites, a cleavage site for subtilisin/kexin-type proprotein convertases, and a cysteine-rich region combined with the absence of any cell adhesion molecule-like protein motif, sets them aside in the family tree. The major characteristic that distinguishes these proteins from other RPTPs is the fact that their single phosphatase domain displays no enzymatic activity whatsoever. Many RPTPs contain catalytically inactive phosphatase domains in their membrane distal ends (D2) but always in combination with an enzymatically very potent membrane proximal PTP domain (D1). Remarkably, in evolution these D2 domains of the conventional

FIG. 4. The juxtamembrane region of IA-2 displays highest affinity for the cytoplasmic domains of IA-2 and IA-2β. COS1 cells were transiently transfected with different VSV- or GFP-tagged domains alone or in combination. Upon immunoprecipitation of the VSV-tagged proteins from cell lysates, the co-precipitating proteins were detected on Western blot with anti-GFP antiserum (A). Expression levels of the GFP-tagged (B) and the VSV-tagged (C) proteins in the lysates are displayed in the bottom panels. Note that use of the juxtamembrane region results in strongest signals in A and that the overnight incubation results in partial degradation of the GFP-tagged proteins (compare panels A and B).

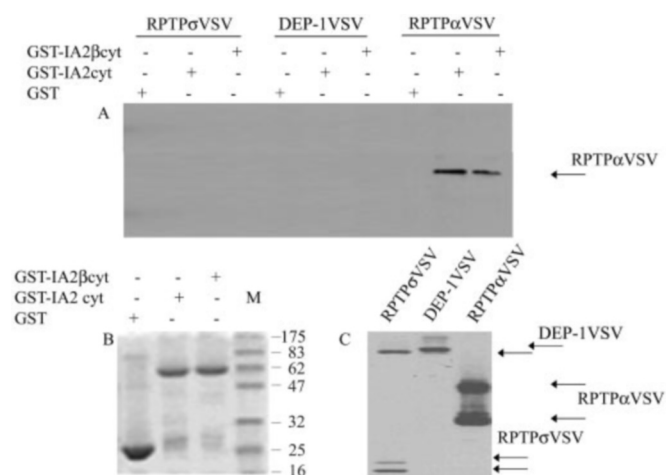
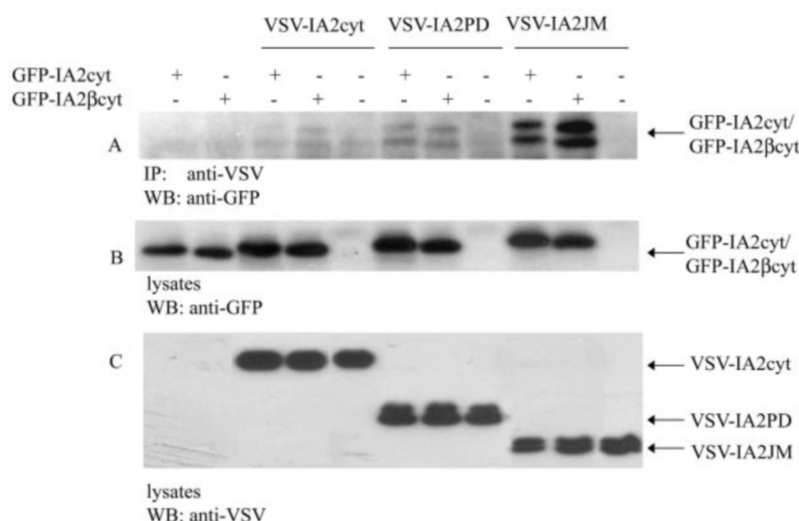


FIG. 5. Interaction of the cytoplasmic domains of IA-2 and IA-2β with RPTPα. Bacterially expressed GST, GST-IA2cyt, or GST-IA2βcyt were used in pull-down assays on lysates of transiently transfected cells expressing VSV-tagged full-length RPTPα, RPTPσ, or DEP-1. Sizes of the bacterially expressed proteins (B) and expression levels of the VSV-tagged proteins (C) are shown in the bottom panels. The two low molecular weight bands visible in the RPTPσ lane correspond to the shed forms (56). For RPTPα both the heavily glycosylated and the non-glycosylated form are present in the lysate (C) but only the non-glycosylated form associates detectably with the GST fusion proteins, as witnessed by Western blotting (A). Size markers are indicated on the right in B.

RPTPs are highly conserved, up to and sometimes even exceeding the level as observed for their active amino-terminal domains. Also, IA-2 and IA-2β are extremely well conserved, recently underscored by the identification of homologous proteins in *Caenorhabditis elegans*, *Drosophila*, and Zebrafish (48, 49).²

To explain this conservation one could envision that D2 domains may have phosphatase activity toward specific, still unidentified substrates. For IA-2 and IA-2β, this is rather unlikely in view of the substitution of several essential amino acid residues within their PTP-like domain. Alternatively, the putative enzymatic activity may be quite different from dephosphorylating phosphotyrosine residues. Sequence alignments reveal that, besides the homology to PTPs, there is an even higher similarity to the myotubularin family of protein-tyrosine phosphatases and the tumor suppressor PTEN in the core region of the IA-2 and IA-2β PTP domain. For PTEN and

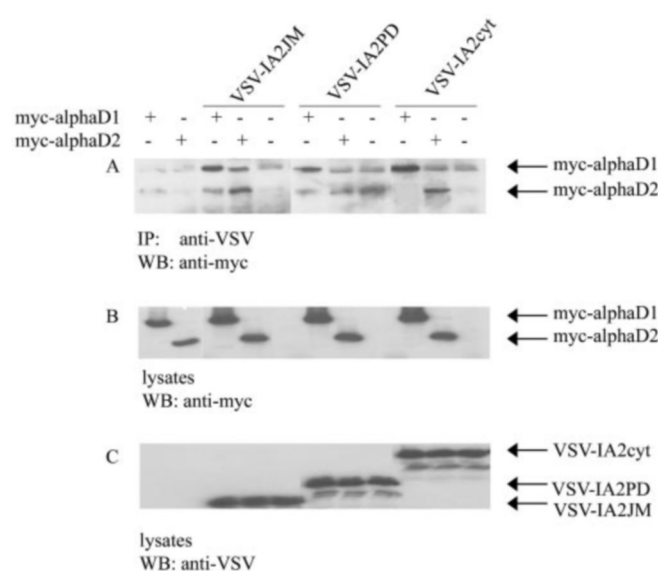


FIG. 6. Binding of IA-2 to PTP domains in RPTPα. COS1 cells were transiently transfected with the VSV-tagged cytoplasmic domains of IA-2 or IA-2β, the myc-tagged catalytically active D1 phosphatase domain of RPTPα that included the so-called wedge or its enzymatic inactive D2 domain in various combinations. VSV-tagged proteins were immunoprecipitated from cell lysates, and co-precipitating proteins were detected with polyvalent anti-myc antiserum (A). Expression levels of myc-tagged proteins in the lysates (B) and the amount of precipitated VSV-tagged proteins (C, blot A reprobbed with polyvalent VSV antiserum) are shown in the bottom panels.

the myotubularins a PtdIns-3,4,5-P₃ (PT(3)P) phosphatase activity was reported (50, 51), but such an activity for IA-2 and IA-2β is at the moment is purely speculative. Also, the IA-2 and IA-2β cytoplasmic domains may serve an anchoring or scaffolding role that is independent of tyrosine phosphorylation. Recently, the binding of IA-2 to the PDZ domains in β2-syntrophin and neuronal nitric-oxide synthase as well as to a novel member of the spectrin protein family was reported (35, 37). Additional experiments provided a model in which stimulation of insulin secretion leads to increased mobilization of secretory granules by inducing dissociation of IA-2 from syntrophin complexes and cleavage of IA-2 by calpain (36).

Yet there exist two additional, appealing alternative functions for IA-2 and IA-2β that directly relate to their PTP-like structure. First of all, the IA-2 and IA-2β cytoplasmic moieties may serve merely as phosphotyrosine recognition and binding motifs and as such extend the collection of protein-signaling

² J. den Hertog, unpublished results.

FIG. 7. Interaction between IA-2 and RPTP ϵ /RPTP μ . COS1 cells were transiently transfected with the VSV-tagged juxtamembrane region, phosphatase domain, or the entire cytoplasmic region of IA-2 with or without various myc-tagged PTP domain combinations from RPTP ϵ and RPTP μ . IA-2 protein parts were immunoprecipitated from cell lysates with anti-VSV antibody P5D4, and co-precipitating proteins were visualized with polyvalent anti-myc antiserum (A). Sizes and levels of the myc-tagged RPTP ϵ and RPTP μ proteins in the lysates (B) and the precipitated VSV-tagged IA-2 proteins (C, blot A reprobed with polyvalent VSV antiserum) are displayed in the bottom panels. The blots shown here represent the result of three independent experiments.

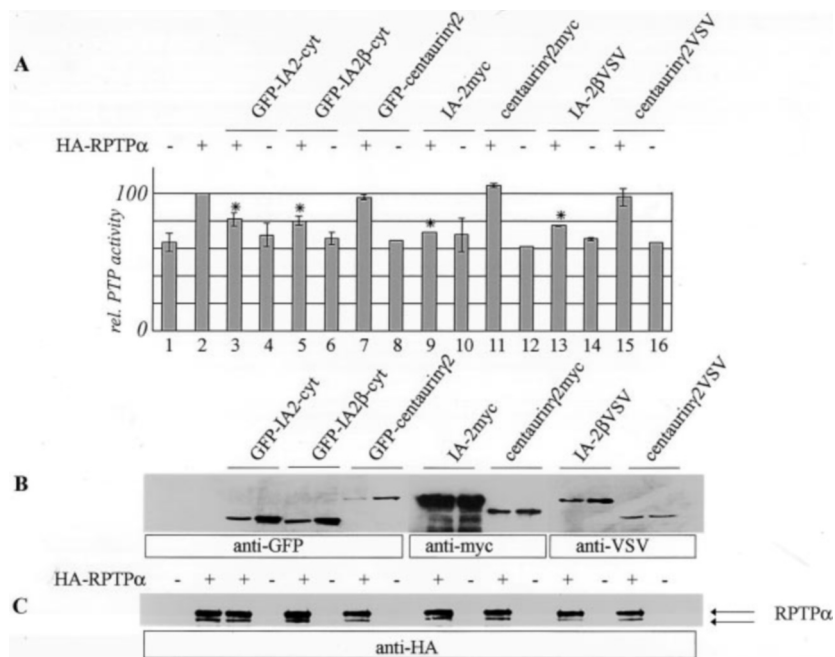
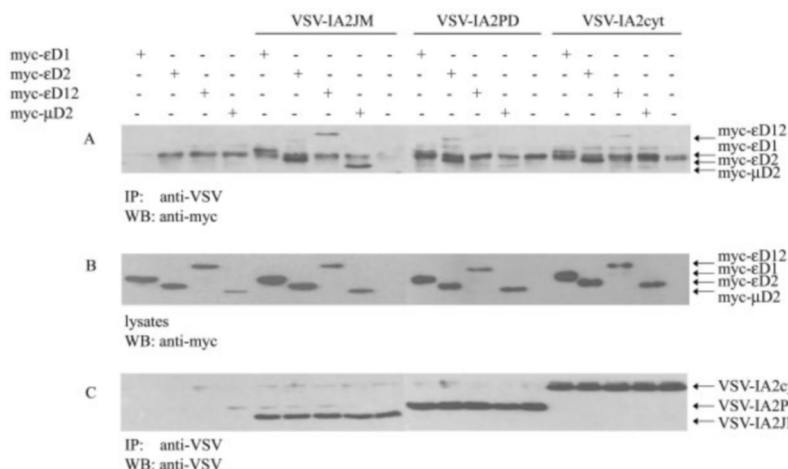


FIG. 8. Inhibition of RPTP α phosphatase activity by IA-2 and IA-2 β . COS1 cells were transiently transfected with VSV-tagged RPTP α with or without tagged IA-2, IA-2 β , and centaurin- γ 2 expression constructs. Lysates were used for Western blotting to determine sizes and levels of the overexpressed tagged proteins (B and C). Lysates were also used to determine protein-tyrosine phosphatase activity using pNPP as a substrate (A) and to assess total protein content for normalization purposes. Results of four independent experiments were collected and used. Equal amounts of RPTP α were found to be present in lysates of transfected cells (C; one representative result is shown). Co-expression with IA-2 or IA-2 β , but not with control centaurin- γ 2, proteins significantly decreased the RPTP α phosphatase activity ($p < 0.05$; indicated by asterisks) as compared with that observed in lysates from cells expressing RPTP α alone, which was set to 100%.

modules that have this capability (SH2 and PTB domains) (52). Finally, it may be that, reasoning very much along the line for inactive D2 domains, IA-2 and IA-2 β actually regulate the substrate specificity and/or activity of other PTPs.

Although considerable progress has been made, little is known about the regulation of PTP activity. For the cytosolic PTPs, protein modules like the SH2, SH3, FERM, or PDZ domains control the activity by linking them to their specific substrates or to defined cellular compartments (53). For the transmembrane PTPs their extracellular domains are potential regulatory regions through binding of cell surface proteins on opposing cells or of soluble ligands. Evidence that ligand-induced dimerization can regulate PTP activity comes from studies on RPTP α and CD45 (8–12, 54). Initiated by crystal structural data on RPTP α D1 domain dimers (8), several studies have revealed that a so-called inhibitory wedge in the RPTP α juxtamembrane region causes inactivation of phosphatase activity upon dimerization (10, 54). For CD45 a similar mechanism is apparent (9, 55), and recently a knock-in mouse model demonstrated the *in vivo* effects of inactivating mutations in the wedge region for protein activity (12). In addition, it has been proposed that for RPTP α the D2 domain might regulate the dimerization state by directly binding to D1 with concomitant inhibition of phosphatase activity (14). We found that

IA-2 and IA-2 β interact with the RPTP α D2 domain independent of a wedge structure. It is tempting to speculate that IA-2 or IA-2 β binding to RPTP α -D2 may change its conformation leading to effects that are reminiscent of the oxidative stress-induced inactivation of RPTP α dimers (17). The IA-2-IA-2 β juxtamembrane region mainly contributed for the binding to RPTP α / ϵ/μ (Fig. 6 and 7). This part is also capable of efficiently binding the inactive phosphatase domain of either IA-2 or IA-2 β itself (Fig. 4). Fine-tuning of homo-/heterodimerization of IA-2-IA-2 β and their association with other RPTPs thus could be an important determinant in controlling the activity of RPTP complexes. Indeed, by exploiting lysates from cells overexpressing IA-2 or IA-2 β in combination with RPTP α , we could show that at least *in vitro* these PTP-like molecules can regulate RPTP phosphatase activity (Fig. 8).

The homodimerization potential of IA-2 and IA-2 β may explain why the affinity for RPTPs appears moderate and the effect on RPTP α activity is limited to some 50% reduction. Part of the protein segments that have an epitope tag in common will inevitably have formed dimers by themselves and thus will not contribute to the detection of co-precipitating proteins or of altered PTP activity. Interestingly, the relative amount of co-precipitated proteins is quite high in the case that full-length IA-2 and IA-2 β proteins were used (Fig. 3), and these also were

more effective than the cytosolic constructs in reducing the RPTP α -derived phosphatase activity (Fig. 8). Several explanations may account for this. Perhaps the extracellular region and the transmembrane domain contribute to the formation of dimers, in line with findings for RPTP α (54), and even the involvement of an up to now unidentified ligand could be envisioned. In addition, for the full-length transmembrane proteins the effective concentration is much higher, and their freedom of movement is reduced, which in combination may favor dimerization.

The discovery of IA-2 and IA-2 β as autoantigens in insulin-dependent diabetes mellitus has led to a wealth of information concerning the immunological aspects and diagnostic use of these molecules. Only recently some data addressing their physiological function have been presented. We here report the formation of IA-2 and IA-2 β homo- and heterodimers as well as their association with other, catalytically active, RPTPs and present evidence for a role of the enzymatically inactive IA-2 and IA-2 β proteins in the regulation of RPTP phosphatase activity.

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