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Interaction between the p21^{ras} GTPase Activating Protein and the Insulin Receptor*

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Gijsbertus J. Pronk‡, René H. Medema‡, Boudewijn M. Th. Burgering‡, Robin Clark§, Frank McCormick§, and Johannes L. Bos‡¶

From the ‡Laboratory for Physiological Chemistry, University of Utrecht, Vondellaan 24A, 3521 GG Utrecht, The Netherlands and §the Department of Molecular Biology, Onyx Pharmaceuticals, Richmond, California 94806

We investigated the involvement of the p21^{ras}-GTPase activating protein (GAP) in insulin-induced signal transduction. In cells overexpressing the insulin receptor, we did not observe association between GAP and the insulin receptor after insulin treatment nor the phosphorylation of GAP on tyrosine residues. However, after insulin treatment in the presence of the phosphotyrosine phosphatase inhibitor phenylarsine oxide (PAO), 5–10% of GAP was found to be associated with the insulin receptor, and, in addition, a fraction of total GAP was phosphorylated on tyrosine. Using *in vitro* binding we showed that the N-terminal part of GAP containing the *src*-homology domains 2 and 3 (SH2-SH3-SH2 region) is involved in binding to the autophosphorylated insulin receptor β-chain. *In vitro* binding between GAP and the autophosphorylated insulin receptor occurred independently of PAO pretreatment. These results suggest that GAP can transiently interact with the insulin receptor after insulin treatment, and this interaction is arrested after PAO pretreatment.

p21^{ras} is a small guanine nucleotide-binding protein which is involved in a number of growth factor-stimulated signal transduction pathways (Hall, 1990). Signaling through p21^{ras} appears to be, at least in part, regulated by the p21^{ras}-GTPase activating protein (GAP).[1] GAP is involved in controlling the level of active GTP-bound p21^{ras} and may be functioning as a downstream target of p21^{ras} as well (Adari *et al.*, 1988; Cales *et al.*, 1988; McCormick, 1989; Sigal *et al.*, 1986; Trahey and McCormick, 1987; Trahey *et al.*, 1988; Vogel *et al.*, 1988; Willumsen *et al.*, 1986; Yatani *et al.*, 1990). Signals that are known to activate p21^{ras} are predominantly elicited by activated tyrosine kinases, like the activated PDGF and EGF receptors, and pp60$^{v\text{-}src}$ (Gibbs *et al.*, 1990; Satoh *et al.*, 1990). In most cases activation of the kinase leads to association of GAP with the active kinase and tyrosine phosphorylation of GAP (Brott *et al.*, 1991; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990; Molloy *et al.*, 1989; Pronk *et al.*, 1992). In addition, GAP is found in complexes with other tyrosine phosphorylated proteins, such as p62 and p190 for which the genes were recently cloned (Ellis *et al.*, 1990; Kaplan *et al.*, 1990; Koch *et al.*, 1991; Moran *et al.*, 1991; Settleman *et al.*, 1992; Wong *et al.*, 1992).

Recently, we showed that p21^{ras} is rapidly activated after stimulation of the insulin receptor, a member of the receptor tyrosine kinase family (Burgering *et al.*, 1991). Upon treatment with insulin, p21^{ras} shifts from the inactive GDP-bound form to the active GTP-bound form in fibroblasts expressing elevated levels of the human insulin receptor. Downward *et al.* (1990) have shown that activation of the T cell receptor in T lymphocytes leads to activation of p21^{ras} as well. For T cell receptor signaling it is suggested that inhibition of GAP activity leads to p21^{ras} activation (Downward *et al.*, 1990; Izquierdo *et al.*, 1992). GAP has two *src*-homology domains 2 (SH2 domains) (Vogel *et al.*, 1988) through which it can interact with activated tyrosine kinases like the PDGF and EGF receptors, pp60$^{v\text{-}src}$, and possibly with the insulin receptor (Anderson *et al.*, 1990; Brott *et al.*, 1991; Ellis *et al.*, 1990; Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990; Margolis *et al.*, 1990; Pronk *et al.*, 1992). Also, GAP is an *in vitro* substrate for the activated insulin receptor kinase (Vogel *et al.*, 1988). Therefore, we focused on the role of GAP in insulin-induced signaling pathways.

In this report, we show that GAP interacts with the insulin receptor after treatment with insulin. This suggests that GAP is involved in insulin-induced signaling pathways.

MATERIALS AND METHODS

Cell Culture and Growth Factor Stimulation—A14 cells are NIH3T3 cells expressing 3×10^5 high affinity human insulin receptors per cell (Burgering *et al.*, 1991). Cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum (GIBCO). For insulin treatment, subconfluent cultures of A14 cells were grown in Dulbecco's modified Eagle's medium, 0.5% fetal calf serum for 18 h to induce quiescence, and insulin (5 μg/ml) was added for 5 min. For PAO pretreatment, cells were incubated with 25 μM PAO for 5 min prior to stimulation with insulin. Pervanadate was prepared by incubating 5 mM sodium orthovanadate in Dulbecco's modified Eagle's medium, pH 7.4, with 1 mM hydrogen peroxide for 15 min at 18 °C. After this period, excess hydrogen peroxide was neutralized by incubation with 200 μg/ml catalase for 15 min at 18 °C. Cells were pretreated with 0.1 mM pervanadate for 5 min, followed by stimulation with insulin.

Cell Lyses and Immunoprecipitation—After treatment, cells were washed twice with ice-cold phosphate-buffered saline, 1 mM sodium orthovanadate. Cells were lysed in lysis buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 0.1 μM aprotinin, 1 μM leupeptin, 1 mM sodium orthovanadate) and lysates were cleared by centrifugation. GAP immunoprecipitation and immunoblotting were performed with a GAP polyclonal antiserum as described earlier (Halenbeck *et al.*, 1990; Pronk *et al.*, 1992). Phosphotyrosine (PY) immunoprecipitations were performed with 10 μg

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¶ To whom correspondence and reprint requests should be addressed. Tel.: 011-31-30-880521; Fax: 011-31-30-888443.

[1] The abbreviations used are: GAP, p21^{ras}-GTPase activating protein; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; SH2, *src* homology domain 2; PY, phosphotyrosine; PAGE, polyacrylamide gel electrophoresis; PAO, phenylarsine oxide.

of a monoclonal antibody against PY (PY20, ICN) at 4 °C for 4 h. After a 2.5-h incubation with the antibody, 4 μl of Protein A-Sepharose were added and incubation was continued for 1.5 h. Immunoprecipitates were washed four times with lysis buffer and once with 10 mM Tris, pH 7.0, 5 mM EDTA. After the last wash, the beads were resuspended in sample buffer (10% glycerol, 60 mM Tris, pH 6.8, 2% SDS, 0.3 M β-mercaptoethanol), incubated at 95 °C for 5 min after which the samples were loaded on a 7.5% SDS-PAGE gel. Immunoblotting was performed as described earlier (Pronk *et al.*, 1992). Anti-insulin receptor immunoprecipitations were performed with an antiserum specific for the insulin receptor α-chain (Amersham Corp.) as described above, with the exception that Protein G-Sepharose was used to collect the immunecomplexes. For insulin receptor precipitations, we also used lentil lectin-Sepharose 4B (Pharmacia LKB Biotechnology Inc.). For these experiments cells were lysed in lentil lectin buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 0.1 μM aprotinin, 1 μM leupeptin, 1 mM sodium orthovanadate). Lysates were cleared by centrifugation and the cleared lysates were incubated with 20 μl of lentil lectin-Sepharose for 30 min at 4 °C. Complexes were washed four times with lentil lectin buffer and once with 20 mM Tris, pH 7.0, and prepared for SDS-PAGE as described above.

In Vitro Kinase Assays—Immunoprecipitations were performed as described above. Immunoprecipitates were washed by four washes with lysis buffer and two washes with kinase buffer (30 mM Tris, pH 7.0, 150 mM NaCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.1 μM aprotinin, 1 μM leupeptin). *In vitro* kinase reactions were performed in 20 μl of kinase buffer at 20 °C for 15 min after the addition of 10 μCi of [γ-^{32}P]ATP (5000 Ci/mmol, Amersham Corp.). Following this incubation, immunoprecipitates were washed three times with lysis buffer and once with 10 mM Tris, 5 mM EDTA, pH 7.0. Samples were further processed as described above. After electroblotting to nitrocellulose, the blot was exposed to x-ray film (Kodak) for 4 h at −70 °C with intensifying screen.

Tryptic Phosphopeptide Analysis—Immunoprecipitations and *in vitro* kinase reactions were performed as described above. Proteins were separated by SDS-PAGE and electroblotted to nitrocellulose. After exposing the blot to x-ray film, bands of interest were cut out for tryptic phosphopeptide analysis. Tryptic phosphopeptide analysis was performed as described earlier (Boyle *et al.*, 1991; Pronk *et al.*, 1992).

In Vitro Binding Assays—A14 cells were treated as described and lysed in lentil lectin buffer. For *in vitro* binding of the GAP SH2-SH3-SH2 region (GAP37 (Martin *et al.*, 1992)) to immobilized proteins, insulin receptor complexes were purified using lentil lectin-Sepharose, separated by SDS-PAGE, and electroblotted to nitrocellulose. Filters were blocked with 2% skimmed milk, 0.5% bovine serum albumin in TPBS (phosphate-buffered saline, 0.1% Tween 20) after which they were incubated with 0.5 μg/ml GAP37 in TPBS, 0.1% bovine serum albumin for 16 h at 4 °C. GAP37 binding was detected by immunoblotting with anti-GAP serum as described.

RESULTS

GAP Is Present in Insulin Receptor Immunoprecipitates—To investigate whether GAP could be found in a complex with the activated insulin receptor, insulin receptor-overexpressing NIH3T3 cells (A14) were stimulated with insulin for 5 min after which the cells were collected and lysed. Insulin receptors with complexing proteins were immunoprecipitated from the lysates and analyzed for the presence of GAP by immunoblotting. Under these conditions, we were not able to detect GAP in the insulin receptor immunoprecipitates (Fig. 1, *upper panel, first two lanes*).

Treatment with phosphatase inhibitors prior to growth factor stimulation can affect certain growth factor-induced effects. For instance, EGF-induced tyrosine phosphorylation of GAP is significantly increased upon pretreatment of the cells with vanadate, a phosphotyrosine (PY) phosphatase inhibitor (Liu and Pawson, 1991). Furthermore, it has been shown that phenylarsine oxide (PAO), another PY phosphatase inhibitor (Garcia-Morales *et al.*, 1990), reveals potential substrates for the activated insulin receptor tyrosine kinase (Frost *et al.*, 1987; Levenson and Blackshear, 1989). Therefore, A14 cells were treated with insulin in the presence of PAO or pervanadate and insulin receptor immunoprecipitates were analyzed for the presence of GAP. In this way we could detect insulin-induced association of GAP to the insulin receptor complex after PAO pretreatment, but not after pervanadate pretreatment (Fig. 1, *upper panel*). Binding of GAP to the insulin receptor complex was also observed after PAO treatment in the absence of insulin.

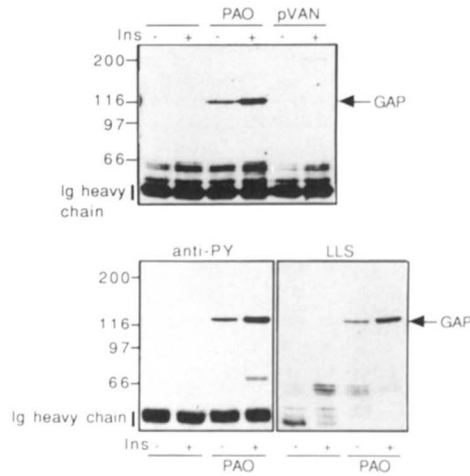

FIG. 1. **GAP in insulin receptor complexes.** *Upper panel*, GAP immunoblot of insulin receptor immunoprecipitates from A14 cells treated with insulin in the presence of phosphatase inhibitors: A14 cells were stimulated with insulin as indicated after; no pretreatment (*left two lanes*), a 5-min pretreatment with PAO (*two middle lanes*) or pervanadate (*right two lanes*). Insulin receptor complexes were immunoprecipitated as described and analyzed for the presence of GAP by immunoblotting. The position of GAP is indicated by the *arrow*. *Lower panel*, GAP immunoblot of PY immunoprecipitates and glycoprotein precipitates from A14 cells treated with insulin in the presence of PAO: A14 cells were treated as described. PY-containing proteins were immunoprecipitated with PY20 and analyzed for the presence of GAP by immunoblotting (*left panel*). Similarly, glycoproteins were precipitated with lentil lectin-Sepharose (*LLS*), and analyzed for the presence of GAP (*right panel*). The GAP protein is indicated by the *arrow*.

To support these results, two other procedures were used to collect the insulin receptor complex. First, antibodies against phosphotyrosine (anti-PY) which bind the autophosphorylated insulin receptor β-chain and second, lentil lectin-Sepharose, which binds the insulin receptor through the glycosyl groups attached to the insulin receptor α-chain. Both procedures were very efficient in collecting insulin receptors as was verified by immunoblotting (not shown). In both cases, GAP was recovered together with the insulin receptor, as detected by GAP immunoblotting (Fig. 1, *lower panel*).

To determine the percentage of GAP that binds to the insulin receptor complex after PAO/insulin treatment, we compared the amount of GAP in GAP immunoprecipitates with the amounts found in insulin receptor and PY immunoprecipitates and after collection of the insulin receptor with lentil lectin-Sepharose. To quantitate the amount of GAP accurately, serial dilutions of the GAP immunoprecipitate were also included (Fig. 2). About 4–5% of GAP is found in insulin receptor immunoprecipitates after PAO/insulin treatment. In PY immunoprecipitates and with lentil lectin-Sepharose the percentages of coprecipitated GAP are higher (20–25% and 8–10%, respectively). In our hands lentil lectin-Sepharose is 2-fold more efficient in collecting insulin receptor complexes than our insulin receptor antibody (not shown). Therefore, we estimate that 5–10% of GAP is bound to insulin receptor complexes after PAO/insulin treatment. The per-

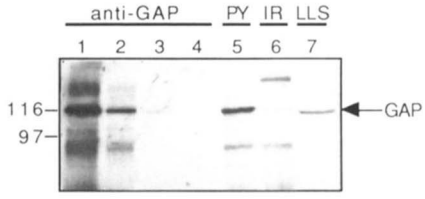

FIG. 2. **Quantification of relative GAP amounts associated with insulin receptor complexes.** A14 cells were treated with PAO and insulin. Cells were lysed and GAP (*lanes 1–4, GAP*), PY (*lane 5, PY*), and insulin receptor immunoprecipitates (*lane 6, IR*), and glycoprotein precipitates using lentil lectin-Sepharose (*lane 7, LLS*) were isolated as described in the text. For accurate quantification, different amounts of the GAP immunoprecipitate were used as "GAP-standards" (1, 0.2, 0.04, and 0.008 volume of lysate used (*lanes 1–4*, respectively)). GAP was detected by immunoblotting with a GAP antiserum as described. The position of GAP is indicated by the *arrow*.

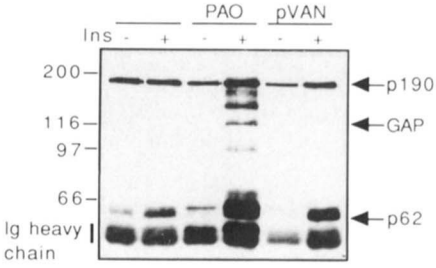

FIG. 3. **Tyrosine phosphorylation of GAP complexes after treatment with PAO and insulin.** A14 cells were stimulated with insulin as indicated after no pretreatment (*left two lanes*), a 5-min pretreatment with PAO (*two middle lanes*), or pervanadate (*right two lanes*). GAP complexes were immunoprecipitated and analyzed for tyrosine-phosphorylation levels by immunoblotting using a monoclonal antibody against PY. Positions of GAP and the GAP-associated phosphoproteins p190 and p62 are indicated by *arrows*.

centage of GAP found in PY immunoprecipitates is even higher than that found using lentil lectin-Sepharose, which is most likely due to phosphorylation of GAP and association of GAP to other tyrosine phosphorylated proteins (see also Fig. 3).

Insulin Receptor β-Chain Is Present in GAP Immunoprecipitates—To investigate whether the insulin receptor is present in GAP immunoprecipitates, we precipitated GAP from A14 cells that were treated with PAO/insulin. GAP immunoprecipitates were analyzed for the presence of the insulin receptor by immunoblotting with polyclonal antisera raised against the insulin receptor. However, we could not detect the insulin receptor in GAP immunoprecipitates, possibly due to the relatively small amount of insulin receptor coprecipitating with GAP and the poor performance of the insulin receptor antisera in immunoblotting (not shown).

To show that the insulin receptor coprecipitates with GAP after PAO/insulin treatment, GAP immunoprecipitates were immunoblotted with PY antibodies. Clearly, two major bands of 62 and 190 kDa coprecipitate with GAP under all conditions (Fig. 3). Interestingly, the tyrosine-phosphorylated p62 levels are higher after insulin treatment, suggesting that insulin treatment affects the interaction between GAP and p62 or increases tyrosine phosphorylation of GAP-associated p62. Both p62 and p190 have been identified as proteins that bind to GAP in a variety of cells, and recently the genes encoding these proteins were cloned, but there exact function remains to be determined (Bouton *et al.*, 1991; Ellis *et al.*, 1990, 1990; Kaplan *et al.*, 1990; Moran *et al.*, 1991; Settleman *et al.*, 1992; Wong *et al.*, 1992). In addition to p62 and p190, a number of other phosphoproteins were observed in the GAP immunoprecipitate after PAO/insulin treatment. The observed 120-kDa phosphoprotein has an electrophoretic mobility similar to that of GAP, as detected by reprobing the blot with GAP antiserum, suggesting that PAO/insulin treatment induces tyrosine phosphorylation of GAP. Another phosphoprotein has a molecular mass of about 96 kDa, and the electrophoretic mobility of this phosphoprotein is identical to that of the 96-kDa autophosphorylated insulin receptor β-chain. To investigate whether the 96-kDa phosphoprotein is the autophosphorylated insulin receptor β-chain, GAP was immunoprecipitated from A14 cells, and *in vitro* kinase reactions were performed on the washed immunoprecipitates. Clearly, there is a significant increase in kinase activity in GAP immunoprecipitates after PAO/insulin treatment (Fig. 4, *upper panel, lane 4*). Interestingly both the 120-kDa and the 96-kDa protein are phosphorylated. Phosphoamino acid analysis of the phosphorylated proteins indicated that the majority of the phosphorylation occurs on tyrosine residues (data not shown). The GAP-associated 96-kDa phosphoprotein has an identical electrophoretic mobility as the *in vivo* and *in vitro* autophosphorylated insulin receptor β-chain (Fig. 4, *lower panel*). This indicates that GAP immunoprecipitates from PAO/insulin-treated cells contain an insulin-inducible tyrosine kinase which is most likely the activated insulin receptor. To show

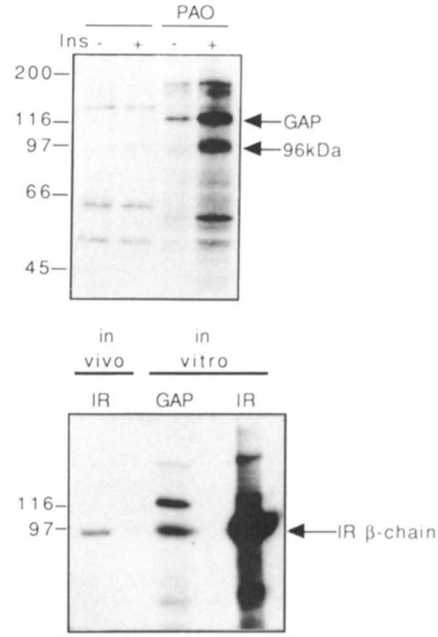

FIG. 4. **The active insulin receptor coprecipitates with GAP complexes.** *Upper panel, in vitro* kinase reactions on GAP immunoprecipitates from A14 cells treated with insulin and/or PAO: A14 cells were not treated (*first lane on the left*), treated with insulin for 5 min (*lane 2*), treated with PAO for 10 min (*lane 3*), or treated with PAO for 5 min prior to stimulation with insulin for 5 min (*lane 4*). GAP immunoprecipitations were performed, and after extensive washing the immune complexes were incubated with [γ-^{32}P]ATP. The positions of GAP and the GAP-associated 96-kDa phosphoprotein are indicated by *arrows*. *Lower panel*, the GAP-associated 96-kDa phosphoprotein has the same electrophoretic mobility as the insulin receptor β-chain: A14 cells were labeled with $^{32}P_i$, treated with PAO and insulin, and, after lysis, insulin receptor complexes were precipitated (*left lane, IR*). For *in vitro* phosphorylations A14 cells were treated with PAO and insulin lysed, and GAP and insulin receptor complexes were immunoprecipitated as described in the text. *In vitro* phosphorylations were performed as described in the text. The *middle lane* shows the GAP immunoprecipitate (*GAP*) and the *right lane* the insulin receptor immunoprecipitate (*IR*). The position of the insulin receptor β-chain is indicated by the *arrow*.

conclusively that the 96-kDa phosphoprotein in GAP immunoprecipitates is the insulin receptor β-chain, tryptic phosphopeptide analysis of *in vitro* phosphorylated proteins was performed. The 96-kDa phosphoprotein from GAP immunoprecipitates and the autophosphorylated insulin receptor β-chain of insulin receptor immunoprecipitates (see Fig. 4, *lower panel*) were digested with trypsine and the phosphopeptides analyzed by two-dimensional thin layer electrophoresis/chromatography. The tryptic phosphopeptide maps of both proteins are similar, although the relative intensities of the different phosphopeptides vary (Fig. 5). We therefore conclude that the 96-kDa phosphoprotein present in GAP immunoprecipitates is the insulin receptor β-chain.

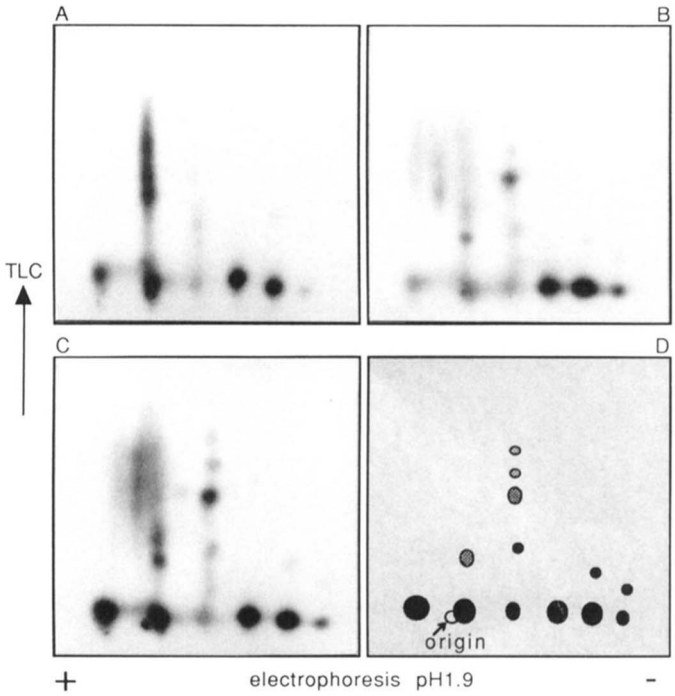

FIG. 5. **Tryptic phosphopeptide maps of the GAP-associated 96-kDa phosphoprotein and the insulin receptor β-chain after *in vitro* phosphorylation.** Shown are tryptic phosphopeptide maps of, respectively, the insulin receptor β-chain (*panel A*), the 96-kDa phosphoprotein from GAP immunoprecipitates (*panel B*), and a mix of the former two (*panel C*). *Panel D* shows the peptides that are common to the two proteins in *black*.

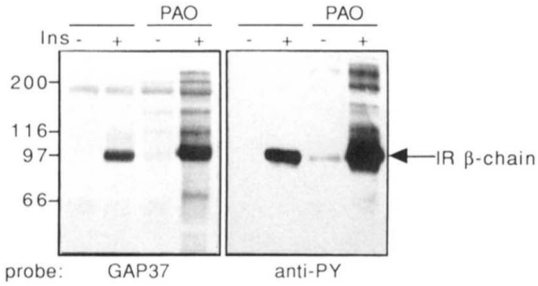

FIG. 6. ***In vitro* binding between the insulin receptor β-chain and GAP SH2 domains dependent on receptor autophosphorylation.** GAP SH2 domains bind to immobilized insulin receptor β-chain (*left panel*): A14 cells were stimulated as described and insulin receptor complexes were purified with lentil lectin-Sepharose as described in the text. Proteins were separated on a 7.5% denaturing gel and electroblotted to nitrocellulose. The blot was incubated with GAP37 (0.5 μg/ml), and GAP37 was detected with a GAP antiserum. The position of the insulin receptor β-chain is indicated by the arrow. GAP binding dependent on insulin receptor β-chain autophosphorylation (*right panel*): insulin receptor complexes were isolated as described above and analyzed for the presence of PY by immunoblotting with PY20 as described in the text.

Taken together, our results show that GAP is associated with the insulin receptor complex in PAO/insulin-treated A14 cells.

SH2-SH3-SH2 Region of GAP Interacts with the Insulin Receptor in Vitro—To investigate whether the association between GAP and the insulin receptor occurs through interactions between the SH2-SH3-SH2 region of GAP and phosphotyrosine residues of the insulin receptor β-chain, we performed *in vitro* binding studies with a recombinant GAP protein containing the SH2-SH3-SH2 region (GAP37) (Martin *et al.*, 1992). Insulin receptor complexes were collected with lentil lectin-Sepharose from A14 cells treated as described. Complexes were separated by SDS-PAGE and electroblotted to nitrocellulose. To detect direct binding of the GAP SH2-SH3-SH2 region to the insulin receptor β-chain the blot was incubated with GAP37 and bound GAP37 was detected with an anti-GAP serum. As shown in Fig. 6 (*left panel*), GAP37 is predominantly bound to a 96-kDa protein. Binding was only observed after the cells were treated with insulin and hence dependent on the presence of the autophosphorylated insulin receptor β-chain, as confirmed by PY immunoblotting (see Fig. 6, *right panel*). Surprisingly, binding was not dependent on PAO treatment as is *in vivo* association between GAP and the insulin receptor. Similar results were obtained with full length GAP, and with insulin receptors collected by immunoprecipitation with antibodies against the insulin receptor. Furthermore, GAP37 copurified with autophosphorylated insulin receptors when it was added to cell lysates before collecting insulin receptors (results not shown). After PAO treatment alone, some insulin receptor β-chain phosphorylation can be detected as well as some GAP37 binding (Fig. 6, *third lane* of each panel). From these results we conclude that the N-terminal part of GAP, containing the SH2-SH3-SH2 region, binds to the insulin receptor β-chain, and that this *in vitro* association is dependent on activation of the insulin receptor β-chain due to insulin treatment.

DISCUSSION

GAP Associates with the Activated Insulin Receptor—We investigated whether GAP associates with the insulin receptor in A14 cells stimulated with insulin (Figs. 1 and 2). After pretreating the cells with the phosphotyrosine phosphatase inhibitor PAO, followed by insulin treatment, approximately 10% of GAP can be recovered in a glycoprotein precipitation, using lentil lectin-Sepharose (Fig. 2). The majority of this GAP appears to be associated with the insulin receptor, because about 4–5% of GAP can be recovered in insulin receptor immunoprecipitations (Fig. 2), and these immunoprecipitations are, in our hands, about 2-fold less efficient in precipitating the insulin receptor, as compared with lentil lectin-Sepharose. Furthermore, a 96-kDa phosphoprotein is present in GAP immunoprecipitates of A14 cells after PAO/insulin treatment (Fig. 3). By way of tryptic phosphopeptide analysis we show that this phosphoprotein is the insulin receptor β-chain (Fig. 5). Finally, *in vitro*, the SH2-SH3-SH2 region of GAP binds to the autophosphorylated insulin receptor β-chain directly (Fig. 6).

In the absence of PAO pretreatment we do not observe *in vivo* association of GAP to the insulin receptor. This suggests that due to PAO pretreatment a transient interaction between GAP and the insulin receptor is stabilized. Previously, it has been shown that PAO pretreatment enhances tyrosine phosphorylations induced by insulin treatment, and hence PAO functions presumably through inhibition of a phosphotyrosine

phosphatase (Frost *et al.*, 1987; Garcia-Morales *et al.*, 1990; Levenson and Blackshear, 1989). However, we do not observe an increase in GAP association after pretreating the cells with pervanadate, a commonly used phosphotyrosine phosphatase inhibitor. This inhibitor increases the association of GAP with the activated PDGF receptor[2] and enhances the phosphorylation of GAP after both PDGF and EGF stimulation (Liu and Pawson, 1991).[2] This suggests that in our case a PAO-sensitive, pervanadate-insensitive phosphotyrosine phosphatase is playing a role in the dissociation of GAP from the insulin receptor. Liao *et al.* (1991) have isolated a PAO-sensitive, pervanadate-insensitive phosphotyrosine phosphatase from 3T3-L1 adipocytes which was able to dephosphorylate pp15, a PAO/insulin-induced phosphoprotein. Whether this phosphatase is involved in enabling dissociation of GAP from the insulin receptor is currently under investigation. Alternatively, the observed PAO effects are not due to the inhibition of a phosphotyrosine phosphatase, but inhibition of another unknown enzymatic activity.

In the absence of insulin, PAO induces a weak phosphorylation of the insulin receptor β-chain (Fig. 6) (Medema *et al.*, 1991), consistent with PAO acting as a phosphotyrosine phosphatase inhibitor. This PAO-induced tyrosine phosphorylation could explain the presence of GAP in insulin receptor precipitates after PAO pretreatment alone. However, it is unlikely that PAO treatment results in novel sites of phosphorylation of the insulin receptor, leading to binding of GAP. This conclusion is supported by the following observations. First, *in vitro*, the GAP SH2-SH3-SH2 region binds to the insulin receptor β-chain that is autophosphorylated in response to insulin treatment without PAO pretreatment. This indicates that insulin treatment by itself is sufficient to induce phosphorylation of the insulin receptor at sites essential for the interaction with the GAP SH2-SH3-SH2 region. Second, tryptic phosphopeptide analysis of the insulin receptor β-chain, after *in vivo* labeling with $^{32}P_i$, revealed no new phosphopeptides after pretreatment with PAO as compared with insulin treatment alone (not shown). These results suggest that the observed effect of PAO is not due to artificial phosphorylations of the insulin receptor. Therefore, the most plausible explanation for our results is that GAP interacts with the insulin receptor after insulin treatment but that this interaction is transient. PAO pretreatment inhibits *in vivo* dissociation of GAP from the insulin receptor, resulting in stable association between GAP and the insulin receptor and as a consequence GAP tyrosine phosphorylation.

Significance of GAP-Insulin Receptor Association—The function of GAP in the regulation of $p21^{ras}$ may be at different levels. First, inhibition of GAP activity may be the trigger to activate $p21^{ras}$ (Downward *et al.*, 1990). Second, GAP may be a negative regulator of $p21^{ras}$, which needs to be activated first by increased GDP/GTP exchange activity (Downward *et al.*, 1990; Huang *et al.*, 1990; Wolfman and Macara, 1990). Finally, evidence exists that GAP is involved in the effector function of $p21^{ras}$ (Martin *et al.*, 1992; Yatani *et al.*, 1990). The results we obtained do not discriminate between these possibilities. The observation that, besides the dramatic increase in $p21^{ras}$GTP, insulin treatment can also induce the interaction between GAP and the insulin receptor suggests that these processes are somehow interconnected. Moreover, PAO treatment not only stabilizes the association of GAP with the insulin receptor, it also inhibits insulin-induced activation of $p21^{ras}$ (Medema *et al.*, 1991). In permeabilized cells we were able to show that insulin-induced activation of $p21^{ras}$ occurs, at least partly, through increase in GTP-binding of $p21^{ras}$, probably due to activation of a guanine nucleotide release factor.[3] Moreover, PAO treatment inhibits this insulin-induced increase in guanine nucleotide exchange.[4] It is possible that the PAO-induced accumulation of GAP-insulin receptor complexes is due to the inhibition of insulin-induced activation of $p21^{ras}$. If this is the case, $p21^{ras}$-GTP may trigger the dissociation of GAP from the insulin receptor. In this view it is interesting to note that PDGF treatment of fibroblasts leads to a relatively low increase in $p21^{ras}$-GTP levels, as compared with insulin treatment of A14 cells, and to association of GAP with the PDGF receptor (Burgering *et al.*, 1991). Alternatively, PAO inhibition of insulin-induced activation of $p21^{ras}$ and the association between GAP and the insulin receptor are separate events. This is currently under investigation.

Whatever the exact mechanism of PAO action is, our results do suggest that GAP is involved in insulin-induced signaling. The use of PAO to reveal a protein complex that is most likely taking part in the normal signaling process puts us in a position to further investigate how the GAP-insulin receptor complex contributes to insulin receptor signaling through $p21^{ras}$.

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