The HMG-I/Y-related protein p8 binds to p300 and Pax2 trans-activation-domain interacting protein to regulate the trans-activation activity of the Pax2A and Pax2B transcription factors on the glucagon gene promoter

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Abstract

p8 is a nuclear DNA-binding protein identified because its expression is strongly activated in response to several stresses. Biochemical and biophysical studies revealed that, in spite of a weak sequence homology, p8 is a HMG-I/Y-like protein, suggesting that p8 may be involved in transcription regulation. Results reported here strongly support this hypothesis. Using a pull-down approach we found that p8 interacts with the general co-activator p300. We also found that, like the HMG proteins, p300 is able to acetylate recombinant p8 in vitro, although the significance of such modification remains to be determined. Then a screening by the two hybrid system, with p8 as bait, allowed us to identify the Pax2 trans-activation-domain interacting protein (PTIP) as another partner of p8. Transient transfection studies revealed that PTIP is a strong inhibitor of the trans-activation activities of Pax2A and Pax2B on the glucagon gene promoter, chosen as model because it is a target of the Pax2A and Pax2B transcription factors. This effect is completely abolished by co-transfection of p8 in glucagon-producing InRIG9 cells, indicating that p8 binding to PTIP prevents inhibition of the glucagon gene promoter. This was not observed in NIH3T3 fibroblasts, that do not express glucagon. Finally, expression of p8 enhances the effect of p300 on Pax2A and Pax2B trans-activation of the glucagon gene promoter. These observations suggest that, in glucagon-producing cells, p8 is a positive co-factor of the activation of the glucagon gene promoter by Pax2A and Pax2B, both by recruiting the p300 cofactor to increase the Pax2A and Pax2B activities and by binding the Pax2 interacting protein PTIP to suppress its inhibition.
Introduction

p8 was cloned because the strong induction of its expression in pancreatic acinar cells during the acute phase of pancreatitis suggested that it was an important regulatory gene (1) and further experiments have shown that p8 activation was not restricted to pancreatic cells. In vivo, p8 mRNA expression is activated in several tissues in response to systemic LPS (2) and in vitro studies showed that a variety of cell lines exhibited transient p8 mRNA expression in response to several stress agents (1, 3, 4) through a p38-dependent pathway (4). However, like other pancreatitis-induced proteins, p8 is also constitutively expressed in several tissues (1, 3). Concomitant studies by another laboratory (5) demonstrated that expression of the candidate of metastasis 1 (Com 1) protein, which is identical to the human p8 (3), could mediate the growth of tumour cells after metastatic establishment in a secondary organ, suggesting that activated expression of p8 in metastatic cells is required for tumour progression. This was recently confirmed by showing that p8-expressing fibroblasts transduced with a retrovirus encoding both the rasV12 and the E1A oncogenes could generate tumours in vivo, whereas p8-deficient fibroblasts could not (6). p8 is, therefore, a stress-induced protein critical for tumour development.

The deduced protein sequence revealed that p8 contains a canonical bipartite signal for nuclear targeting, suggesting that p8 should be located in the nucleus. Supporting this hypothesis we detected p8 within the nucleus of Cos-7 cells transfected with a p8 expression plasmid, although it was also partly located to the cytoplasm (3). Furthermore, analysis of the p8 primary structure suggested that it was a DNA-binding protein (1). More recently, we performed biochemical and biophysical studies showing that human recombinant p8 was in many aspects very similar to the HMG-I/Y proteins, although sharing with them only 35% aminoacid sequence identity (7). Thus, in spite of a weak sequence homology, p8 can be considered as a HMG-I/Y-like protein. In addition, we recently found that p8 acts as a co-transcription factor since it is able to enhance the transcription activity of the Smad proteins in response to TGF-β (8). The mechanism by which p8 could enhance the trans-activation of Smads remains however unclear.

This paper describes first attempts to characterize partners of p8 in gene regulation. We found that p8 binds to and is acetylated by p300, and when looking for other partners of p8 by yeast two-hybrid screening, we observed strong interaction with the Pax2 trans-activation-domain...
interacting protein PTIP. Finally, we also demonstrated on the model of the glucagon gene promoter that these interactions are functionally active.

Materials and Methods

Pull-down of p300 by p8
The expression vector pQE-30 (Qiagen) containing the human p8 sequence cloned in its BamHI-HindIII site was used to generate a fusion protein with an amino-terminal histidine tag to the human p8 sequence. The protein was produced and purified on nickel-agarose resin (Qiagen) as previously reported (7). The recombinant protein was named His6hp8. Approximately 20 µl bed volume of nickel-agarose (Qiagen) bound to His6hp8 was incubated in 100 µl of pull-down buffer (20 mM HEPES/KOH, pH 7.5, 100 mM KCl, 5 mM MgCl2, 0.5 mM EDTA, 0.05% Nonidet P-40, 1 mM dithiothreitol, 0.02% bovine serum albumin, and 1 mM PMSF, 10 µg/ml leupeptin and 10 µg/ml aprotinin as protease inhibitors) together with 100 µg of HeLa cell extract. After 3 h of incubation at 4°C, the nickel-agarose beads were washed five times with 1 ml PBS before elution with SDS-PAGE sample buffer. The eluted material was resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and p300 was detected with a specific polyclonal antibody which does not cross-react with CBP (N-15, Santa Cruz Biotechnology).

Immunoprecipitation of p300 and p8 acetylation test
For immunoprecipitation, about 5 x 10^6 Cos-7 cells, seeded into 15-cm dishes, were transfected with 5 µg of the pCMVbp300-CHA plasmid (9) using the Fugene reagent (Roche Molecular Biochemicals). Cells were harvested 24 h later and lysed in 500 µl of 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM MgCl2, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM PMSF, 10 µg/ml leupeptin and 10 µg/ml aprotinin on ice for 30 min. After clearing the lysate containing the p300-CHA hybrid protein, one half was used for immunoprecipitation with 0.5 µg of anti-HA antibody (3F10, Roche Molecular Biochemicals) for 4 h at 4°C in the presence of 50 µl bed volume of protein G preincubated in cell lysates from non-transfected cells supplemented with 1% bovine serum albumin. The remaining half of the lysate was used as a control in which the anti-HA antibody was omitted. Immunoprecipitates were washed four times with 1 ml of lysis buffer and one time with 2X TBE buffer (89 mM Tris, pH 8.3, 89 mM sodium borate, 1 mM EDTA). One tenth of the material bound to the beads was eluted in SDS-loading buffer and analyzed by Western blot using a specific antibody to p300 (N-15) and the ECL detection system (Amersham Pharmacia). Another tenth was used to test its
capacity to acetylate $\text{His}^6\text{hp8}$ in vitro. The reaction was performed in TBE, 1 mM dithiothreitol buffer, with 0.1 mg/ml protein substrate (as indicated in each case) and 18 µM [1-14C]acetyl-CoA. The standard assay was performed at 25°C for 30 min and initiated by adding the protein substrate to the mixture containing the acetyltransferase and acety-CoA in the relevant buffer. Histone from calf thymus (Roche Molecular Biochemicals) was used as a positive control. Reaction products were separated by SDS-PAGE and 14C-labeled proteins visualized by autoradiography.

**Yeast two-hybrid screen**

The Matchmaker two hybrid system (Clontech Laboratories) was used according to the protocols provided by the manufacturer. Using polymerase chain reaction-based strategies, we subcloned the complete coding sequence of the human p8 (3) into the BamHI-SalI site of the pGBT9 vector to generate a fusion protein with the GAL4 DNA binding domain (BDhp8). That protein was used as bait to screen a HeLa cell cDNA library constructed in the pACT2 vector to generate fusion products with the GAL4 activation domain. This library was also purchased from Clontech Laboratories. Yeast cells were initially selected for growth on -His plates. Of the about 1 x 10^6 transformants screened, 23 grew well on -His plates (colonies >2mm). Plasmid DNAs were isolated from each one. To identify false positives and self-activators, the plasmids were reintroduced into the original yeast strain expressing no BDhp8. Nine independent clones were obtained and sequenced. Sequence corresponded to the human counterpart of the mouse PTIP (10).

**In vitro binding of MBP-hPTIP to $\text{His}^6\text{hp8}$**

hPTIP cDNA encoding residues 773 to 1056 was amplified by PCR and subcloned into the SalI restriction site of the pMAL-c2 vector (New England Biolabs), fusing the maltose binding protein (MBP) with the carboxyl-terminal region of the hPTIP. The fusion protein, named MBP-hPTIP, was produced following the manufacturer’s recommendations. To test the $\text{His}^6\text{hp8}$-hPTIP interaction in vitro, we incubated 1 µg of the purified MBP-hPTIP or MBP (as a control) in the presence of 2 µl (about 2 µg) of the $\text{His}^6\text{hp8}$ bound to nickel beads or only free nickel beads (as a control) in 25 µl of a binding buffer containing 20 mM Hepes pH 7.4, 200 mM NaCl, 0.25 mM MgCl$_2$, 0.5 mM DTT, 1% NP40 during 1 h at room temperature. The nickel beads were recovered by centrifugation, and washed five times with the binding buffer and once with PBS prior to elution with SDS-PAGE sample buffer. The eluted material was resolved on SDS-PAGE, transferred onto nitrocellulose membrane, and MBP-hPTIP and
MBP proteins were detected by Western blotting with a specific MBP antibody (New England Biolabs).

**Trans-activation Assays**

Expression vectors containing Pax2A and Pax2B (11), the hp8 expression vector (3), the reporter plasmid -138GluCAT (12) and the pMYC-PTIP (10) were previously reported. The pCMVbp300-CHA plasmid (9) was a gift from A. Hecht (Max Planck Institute of Immunology, Freiburg). InRIG9 and NIH3T3 cells were grown as described (1, 12). Cells were transfected with the Fugene reagent following recommendations of the supplier (Roche Molecular Biochemicals). The pCMV/βgal plasmid encoding β-galactosidase was added to monitor transfection efficiency. Cell extracts were prepared 48 h after transfection and analyzed for CAT and β-galactosidase activities as previously described (8). A minimum of three independent transfections were performed; each of them was carried out in duplicate or triplicate.

**Results and Discussion**

p8 interacts with the general co-activator p300 in vitro

Because p8 is a nuclear protein (3) with DNA binding ability (7) that seems to act as a co-transcriptional factor since it is structurally related to the HMG-I/Y (7) and its expression enhances the Smad trans-acting activity (8), we began its functional study by examining a relationship with the general co-activator of transcription p300. In fact, p300 is coupled to the basal transcription machinery and acts as a general co-activator of several transcription factors (13). *In vitro* pull-down analysis were performed by adding His6hp8 to HeLa extracts containing p300 and monitoring formation of a p8-p300 complex. As shown in Figure 1, a significant binding of p8 to p300 was indeed found.

p8 is acetylated by p300 in vitro

Then, because p8 is a HMG-I/Y-related protein and HMG-I/Y is acetylated by p300 (14), we examined whether p8 is also acetylated by p300. We therefore tested the ability of p300 to incorporate radioactive acetate into His6hp8. Figure 2 demonstrates that the immunoprecipitated recombinant human p300 efficiently acetylates His6hp8. The acetylation is specific since bovine serum albumin, which also has a high lysine content, was not acetylated (data not shown). Nucleosomal histones served as positive control. The labeling is enzyme dependent since reaction mixtures containing [1-14C]acetyl-CoA but lacking enzyme failed to
incorporate acetate into $^{\text{His}}_6$hp8. We conclude that p8 is specifically acetylated by p300 (Figure 2).

p8 binds the Pax2 trans-activation-domain interacting protein PTIP
Previous suggestion that the p8 protein is a general co-transcription factor was based on biochemical and biophysical similarities of p8 with HMG-I/Y proteins and its ability to bind DNA in a sequence-independent manner. In that context, one should expect that protein factors bind p8 to confer some specificity to the resulting transcription regulation complex. In order to identify such protein factors, a HeLa cell cDNA library was screened with a yeast two-hybrid system, using the complete coding sequence of human p8 as bait. A cDNA fragment, encoding a protein that specifically interacted with p8 in yeast, was isolated. Comparison of its sequence with sequences in GenBank revealed significant similarities with a previously reported mouse cDNA corresponding to PTIP, a BRCT domain-containing gene that interacts with the transcription factor Pax2 (10). The 283 amino-acid carboxyl-terminal region of human PTIP showed 89% identity with the mouse counterpart (Figure 3).

The interaction between human PTIP and p8 observed in yeast was confirmed by in vitro studies using a pull-down analysis with the $^{\text{His}}_6$hp8 and the MBP-hPTIP products (Figure 4). As expected, $^{\text{His}}_6$hp8 strongly interacted with recombinant MBP-hPTIP but not with MBP alone.

p8 reverses the inhibition by PTIP of the glucagon gene promoter trans-activation by Pax2
PTIP interacts with the Pax2 transcription factor, is expressed ubiquitously and can bind active chromatin (10). These observations strongly suggest that PTIP is a co-factor of the regulation of transcription by Pax2. Following our observation that PTIP interacts with the HMG-I/Y-related protein p8, we made the hypothesis that the resulting PTIP-p8 complex could regulate the trans-activation activity of Pax2. To check that possibility, we transfected the glucagon-producing InRIG9 cells with i/ a vector harbouring the region between nt -138 and +58 of the glucagon promoter, which contains the Pax2-responsive G1 element (12), driving the CAT reporter gene and ii/ expression vectors coding for Pax2A and Pax2B. Pax2A and Pax2B strongly trans-activated the glucagon promoter in the glucagon-producing cell line InRIG9 (Figures 5A and B) as well as in the non-islet-derived cell line (NIH-3T3, Figures 5C and 5D) as previously reported (12). Co-transfection of the PTIP expression plasmid in InRIG9 cells inhibited trans-activation of the reporter gene by Pax2A or Pax2B to 25 and 30% of the full activity, respectively (Figures 5A and B), confirming that PTIP binds
the transcription factor Pax2 to inhibit its *trans*-activation capacity. A similar observation was made in NIH3T3 fibroblasts (Figures 5C and D). However, when either Pax2A or Pax2B was co-transfected with a p8 expression plasmid into InRIG9 cells, the effect of PTIP was completely abolished, indicating that p8 binding to PTIP prevents PTIP inhibition of Pax2 activity (Figures 5A and B). Interestingly, the effect of p8 was not observed in NIH3T3 fibroblasts (Figures 5C and D), suggesting that p8-mediated inhibition of PTIP activity is cell type-specific. These results support the hypothesis that in some cell types p8 can activate Pax2-depending genes upon binding to the Pax2 inhibitor PTIP.

**p300 enhances the effect of p8 on glucagon promoter *trans*-activation by Pax2A and Pax2B**

Results presented above demonstrating association of p300 with p8 led us to test whether p300 could influence regulation by p8 of the glucagon gene promoter activity. This possibility was tested using the Pax2-responsive region of the glucagon gene promoter as target of the Pax2A and Pax2B transcription factors. Further transfection with vectors expressing p300 and p8 allowed monitoring the combined influence of these proteins on the activity of the glucagon gene promoter. As expected (12), Pax2A and Pax2B strongly *trans*-activated the glucagon gene promoter (Figures 6A and B). p300 enhanced Pax2 *trans*-activation activity only weakly, but its co-transfection with p8 significantly increased Pax2 capacity, whereas p8 alone had no significant effect. Similar experiments conducted in non-glucagon producing NIH3T3 fibroblasts showed that expression of p300 also enhanced the Pax2A and Pax2B *trans*-activation activities, but expression of the glucagon promoter remained very low as compared to InRIG9 cells, and co-expression with p8 only led to a small increase (Figures 6C and D). Altogether, these observations strongly suggest that in glucagon-producing cells p8, in association with p300, is a co-activator of Pax2. In this context, p300 contributes to the glucagon gene promoter activation (15).

**Concluding remarks**

We found that the HMG-I/Y-related protein p8 interacts with the general co-activator p300 and that p300 acetylates p8. Relationship between the acetylation of p8 and its transcriptional activity remains to be determined. Then, selection by the two hybrid system allowed us to identify PTIP, a Pax2 binding protein, as another partner of p8. We could demonstrate that PTIP is a strong inhibitor of the Pax2 *trans*-activation activity on the glucagon gene promoter and that p8 binding to the Pax2 inhibitor PTIP prevents inhibition of the glucagon gene promoter. This was observed in glucagon-producing cells only. We suggest that, in glucagon-
producing cells, p8 recruits the p300 cofactor and binds the Pax2 interacting protein PTIP to suppress its inhibitory activity, resulting in Pax2 activation (see Figure 7). This is not the first evidence that p8 can regulate the activity of a transcription factor. We reported previously that p8 interacts with Smad proteins after activation by TGF-β. In fact, fibroblasts derived from homozygous p8 knockouts are partially defective in the Smad signaling of the TGF-β pathway, and this defect is corrected by co-transfection with a p8 mammalian expression vector (8). Interestingly, Smad activity is also, at least in part, p300-dependent (16). Although the mechanism by which p8 enhances the Smad trans-activation activity is not elucidated yet, we suggest that p8 contributes to the regulation of the transcription of some genes by recruiting p300, which in turn may act as an adapter protein linking activators to the basal transcription machinery. In the particular case of glucagon-producing cells, the p8-p300 complex would in addition relieve inhibition of transcription by PTIP.

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References


Legends of Figures

**Figure 1**

*In vitro* interaction between p8 and p300. Pull-down assay was performed to determine the interaction between p8 and p300. In (A) His6p8 bound to nickel-agarose was incubated in pull-down buffer together with HeLa cell extract for 3 h at 4°C. Binding reactions were extensively washed and eluted with SDS-PAGE sample buffer. The eluted material was resolved on SDS-PAGE gel, transferred onto nitrocellulose membrane, and p300 detected with a specific polyclonal antibody which does not cross-react with CBP, whereas p8 was detected with an anti-human p8. In (B) the HeLa cellular extract was omitted, and in (C) it was incubated with nickel-agarose beads without bound His6p8 as a control.

**Figure 2**

Cos-7 cells were transfected with the pCMVbp300-CHA plasmid. Cell extract was prepared 24 hr after transfection. One half was used for immunoprecipitation with anti-HA (3F10) antibody in the presence of protein G-agarose beads and in the remaining half antibody was omitted. One tenth of the material bound was eluted in SDS-loading buffer and analysed by Western blot using the anti-p300 (N-15) antibody and the ECL detection system (lower panel). Another tenth was used to test its capacity to acetylate the His6p8 *in vitro* using [1-14C]acetyl-CoA as a donor of the acetyl group (top panel). Reaction products were separated on SDS-PAGE and 14C-labeled proteins were visualized by autoradiography. Middle panel shows the recombinant His6p8 protein stained with Coomassie blue. Right panel shows p300-CHA acetylated histone as a positive control.

**Figure 3**

Sequence comparison of the carboxyl-terminal regions of the human (hPTIP) and mouse (mPTIP) PTIP. Aminoacid identities are indicated in the consensus sequence shown in the middle and similarities are noted as (+). Aminoacids of mPTIP were numbered according to the Genbank sequence with accession number AF104261, whereas hPTIP numbering was made according to the nucleotide sequence obtained from the cDNA clone identified by the two hybrid system.
Figure 4

*In vitro* interaction between PTIP and p8. To test the \(\text{His}_6\text{hp8}\)-hPTIP interaction *in vitro* we incubated purified MBP-hPTIP in the presence of \(\text{His}_6\text{hp8}\) bound to nickel-agarose beads, during 1 h at room temperature. The nickel-agarose beads were recovered by centrifugation and extensively washed prior to elution with SDS-PAGE sample buffer. The eluted material was resolved by SDS-PAGE and transferred onto nitrocellulose membrane. MBP-hPTIP and MBP proteins were detected by Western blotting with a specific MBP antibody, and p8 with an anti-human p8 antibody. In (A) MBP was incubated with \(\text{His}_6\text{hp8}\) bound to nickel-agarose beads as a control of non-specific interaction between MBP and \(\text{His}_6\text{hp8}\). In (B) \(\text{His}_6\text{hp8}\) was omitted, only free nickel-agarose beads were incubated with MBP. In (C) MBP-hPTIP was incubated with \(\text{His}_6\text{hp8}\) bound to the nickel-agarose beads. In (D) \(\text{His}_6\text{hp8}\) was omitted, only free nickel-agarose beads were incubated with MBP-hPTIP. In (E) and (F) only purified hPTIP and MBP respectively were loaded onto the gel.

Figure 5

p8 expression reverses the effect of PTIP on the *trans*-activation activity of Pax2 on the glucagon gene promoter. InRIG9 (A and B) and NIH3T3 (C and D) cells were transfected with expression plasmids encoding Pax2A (A and C), Pax2B (B and D), PTIP and p8, using the Fugene reagent. To determine the *trans*-activation activity of Pax2, we used the Pax2-responsive region of the glucagon gene promoter as described in the Legend of Figure 3. The pCMV/\(\beta\)gal plasmid encoding \(\beta\)-galactosidase was added to monitor transfection efficiency. Cell extracts were prepared using the reporter lysis buffer 48 hr after transfection and analyzed for CAT and \(\beta\)-galactosidase activities. All experiments were performed with equal amount of plasmids. CAT activity was normalized for transfection efficiency by expressing its activity relative to \(\beta\)-galactosidase activity. The data represent means ± SEM.

Figure 6

Effect of the co-expression of p8 and p300 on *trans*-activations by Pax2A and Pax2B. InRIG9 cells were grown as described in the Legend of Figure 5. InRIG9 (A and B) and NIH3T3 (C and D) cells were transfected with expression plasmids encoding Pax2A (A and C), Pax2B (B and D), p300 and/or p8, using the Fugene reagent. To determine the *trans*-activation activity of Pax2, we used the Pax2-responsive region of the glucagon gene promoter as described in the Legend of Figure 3. The pCMV/\(\beta\)gal plasmid encoding \(\beta\)-galactosidase was added to monitor transfection efficiency. Cell extracts were prepared 48 hr after transfection, using the
reporter lysis buffer, and analyzed for CAT and β-galactosidase expressions. Results were normalized to CAT activity. The data represent means ± SEM.

**Figure 7**

An integrative view of the regulatory mechanism of Pax2, PTIP, p8 and p300 on the glucagon gene promoter. Pax2 activates glucagon gene transcription. PTIP acts negatively on Pax2 and p8 acts negatively on PTIP which relieves Pax2. In addition, p8 recruits the positive factor p300 to the transcription complex.
Figure 1
Figure 2
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Figure 3
Figure 4
Figure 5A

Normalized CAT activity

InRIG9 cells

Pax2A + + + +
PTIP + + + +
p8 + + + +
GluCAT + + + +

A
Figure 5B

Normalized CAT activity

Pax2B  +  +  +  +
PTIP    +  +  +  +
p8      +  +  +  +
GluCAT  +  +  +  +  +  +  +  +  +

InRIG9 cells
Figure 5C

NIH3T3 cells
Figure 5D
Figure 6A

InRIG9 cells

Normalized CAT activity

Pax2A  p300  p8  GluCAT

+  +  +  +
Normalized CAT activity

InRIG9 cells

Figure 6B
Figure 6C

GluCAT

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NIH3T3 cells
Figure 6D

NIH3T3 cells

Pax2B
p300
p8
GluCAT

Normalized CAT activity

NIH3T3 cells
Figure 7
Genes Structure and Regulation:
The HMG-I/Y-related protein p8 binds to p300 and Pax2 trans-activation-domain interacting protein to regulate the trans-activation activity of the Pax2A and Pax2B transcription factors on the glucagon gene promoter

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