ATF-7, a Novel bZIP Protein, Interacts with the PRL-1 Protein-tyrosine Phosphatase*

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We have identified a novel basic leucine zipper (bZIP) protein, designated ATF-7, that physically interacts with the PRL-1 protein-tyrosine phosphatase (PTPase). PRL-1 is a predominantly nuclear, farnesylated PTPase that has been linked to the control of cellular growth and differentiation. This interaction was initially found using the yeast two-hybrid system. ATF-7 is most closely related to members of the ATF/CREB family of bZIP proteins, with highest homology to ATF-4. ATF-7 homodimers can bind specifically to CRE elements. ATF-7 is expressed in a number of different tissues and is expressed in association with differentiation in the Caco-2 cell model of intestinal differentiation. We have confirmed the PRL-1-ATF-7 interaction and mapped the regions of ATF-7 and PRL-1 important for interaction to ATF-7's bZIP region and PRL-1's phosphatase domain. Finally, we have determined that PRL-1 is able to dephosphorylate ATF-7 in vitro. Further insight into ATF-7's precise cellular roles, transcriptional function, and downstream targets are likely to be of importance in understanding the mechanisms underlying the complex processes of maintenance, differentiation, and turnover of epithelial tissues.

It is clear that many cellular processes are regulated through protein phosphorylation. This post-translational modification is responsible for the control of a wide variety of important processes, including the regulation of metabolism, cell proliferation, the cell cycle, gene expression, protein synthesis, and cellular transport (1, 2). Because phosphorylation is a dynamic and reversible process, it follows that phosphatases are as important as kinases in its regulation (3, 4). Phosphorylation of transcription factors and their associated proteins is one of the principal methods used to regulate gene expression (1, 2, 5, 6). Alterations in protein phosphorylation states bring about these changes in a number of different ways, including the regulation of subcellular localization (7–9), changes in DNA binding (10, 11), or alterations in transactivating ability. Classic examples of this latter phenomenon include the basic leucine zipper (bZIP) proteins CREB and c-Jun, where phosphorylation of specific residues in the transactivating domain has been demonstrated to up-regulate transactivation, probably by allowing interaction of these proteins with transcriptional coactivator such as CREB-binding protein (12, 13). Kinases or phosphatases may also, in some situations, bind transcription factors but influence transcription by acting on proteins other than the transcription factors themselves (7, 14). An example of this phenomenon is the nuclear tyrosine kinase c-Abl, which binds to p53 and increases its transactivating ability without phosphorylating it. It is thought that Abl may be able to execute this function by phosphorylating the C-terminal domain of RNA polymerase II, which is known to be extensively phosphorylated on tyrosine (14, 15).

The PRL-1 protein-tyrosine phosphatase (PTPase) was initially identified as an immediate-early response gene in regenerating liver and mitogen-stimulated fibroblasts (16). PRL-1 is a 20-kDa protein that contains the “signature” amino acid sequence for the active site of PTPases but otherwise does not contain regions of homology to any previously described protein (16). PRL-1 is primarily localized to the cell nucleus with a discrete, reproducible “speckled” pattern on immunofluorescence. Under certain circumstances, PRL-1 also is localized to extranuclear sites in the cell (17, 18). PRL-1 is found in the insoluble cellular fraction, despite the fact that it is readily soluble when expressed in bacteria (16). This is likely a result of protein prenylation, because PRL-1 is a farnesylated protein (19). When PRL-1 was stably overexpressed in 3T3 fibroblasts, altered growth characteristics became apparent, including a faster doubling time, growth to a greater saturation density, altered morphology, and evidence of anchorage-independent growth manifested by the ability of these cells to grow in soft agar (16). Overexpression of PRL-1 in epithelial cells resulted in tumor formation in nude mice (19).

PRL-1 is also significantly expressed in intestinal epithelia, and in contrast to PRL-1’s expression pattern in liver, its expression is associated with cellular differentiation in the intestine. Specifically, PRL-1 is expressed in villus but not crypt enterocytes, and in differentia ted, but not proliferating, Caco-2 cells (20). Recently, PRL-1 protein was found to be expressed during development in a number of differentiating epithelial tissues (17). These results suggest that PRL-1 may have divergent roles in different tissues. It is an established feature of some growth response genes that they may play a role in

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†The abbreviations used are: bZIP, basic leucine zipper protein; PTPase, protein-tyrosine phosphatase; CRE, cyclic-AMP response element; CREB, CRE-binding protein; ATF, activating transcription factor; bp, base pair(s); C/EBP, CCAAT enhancer-binding protein; GST, glutathione S-transferase; TK, thymidine kinase; aa, amino acid(s); PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s).
terminal differentiation in some tissues (21–25). The apparently paradoxical dual roles may be explained by the availability of different substrates or cofactors in different cells, different kinetics of protein expression, or by the presence of scaffolding or anchoring proteins that may direct an enzyme to a different cellular location and different substrates (26, 27).

Significant insight into PRL-1’s specific cellular functions and the reasons for its apparently varied expression pattern in different tissues may be derived from identification of PRL-1’s substrates and other cellular partners. To that end, we performed a yeast two-hybrid screen using PRL-1 as bait. We have identified a novel protein that interacts with PRL-1. This protein, which we have designated ATF-7, is a bZIP protein most closely related to members of the ATF/CREB family. We have functionally confirmed that ATF-7 is a bZIP protein by showing that its homodimers specifically bind to cyclic AMP response (CRE) elements. We have confirmed the interaction of PRL-1 and ATF-7 using GST binding and coimmunoprecipitation assays, and we have mapped the sites of interaction to include PRL-1’s phosphatase domain and ATF-7’s bZIP domain. ATF-7 is expressed in a number of different tissues, and it is expressed in association with differentiation in the Caco-2 cell model of intestinal differentiation. Finally, we have determined that PRL-1 is able to dephosphorylate ATF-7 in vitro. It is likely that further insight into ATF-7’s precise cellular roles, transcriptional function, and downstream targets will be of importance in understanding the mechanisms underlying the complex processes of maintenance, differentiation, and turnover of epithelial tissues.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid System and β-Galactosidase Assays—** The N-terminal 132 amino acids of PRL-1 fused to the C terminus of the GAL4 DNA binding domain in the yeast expression vector pGPT9 (CLONTECH) was constructed from the full-length PRL-1 cDNA. This construct contains most of the full-length PRL-1 cDNA except for the C-terminal basic region and CCAAT farnesylase domain. The active site cysteine (Cys-104) was mutated to serine as previously described (C104S) (16). Use of active site cysteine-serine mutant PTPases to demonstrate binding of PTPases to a well-characterized and validated method (28–30). A 373-L1 adipocyte library was synthesized from fully differentiated adipocytes with a cDNA synthesis kit (TECH) (gift of Dr. Alan Saltiel) (31). The yeast strain HF7c was co-transformed with the GAL4-PRL-1 construct and with the 3T3-L1 adipocyte library. The resulting transformants were plated on selection medium lacking tryptophan, leucine, and histidine and were incubated at 30 °C for 4–5 days. 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was exposed to x-ray film. Supershifts were performed by incubating 1–1.5 μl of primary antibody with 10 μg of translated proteins in binding buffer for 45 min at 4 °C, prior to addition of labeled oligonucleotide. The two proteins or protein fragments being tested were translated simultaneously as described above. One protein used was fused to the Myc-tag epitope. 7.5 μl of the in vitro translated protein was incubated with to 5 μl of anti-Myc-tag antibody or control sera in 500 μl of IP buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Triton) for 1 h at 4 °C. Immunocomplexes were bound to protein A-agarose beads and, after washing four times in IP buffer, were resolved by SDS-PAGE and visualized by autoradiography.

**Northern Blots—** RNA preparation, Northern blot analyses, and labeling of recombinant plasmids have been described elsewhere (16, 32). Caco-2 cells were grown and harvested with respect to proliferating and differentiated phenotypes as previously described (20). Total RNA was extracted from cells and from mouse tissues using the techniques previously described (32, 33). Hybridization buffer consisted of 10% dextran sulfate, 40% formamide, 0.06 mM sodium, 7 mM Tris (pH 7.6), 0.8% Denhardt’s solution, and 0.002% heat-denatured, sonicated salmon sperm DNA. Northern blots were hybridized at 42 °C for 16 h and washed for 30 min twice at 60 °C in 0.1% SDS at pH 7.0.

**GST Binding Assays—** Radiolabeled in vitro translated proteins (5 μl) were incubated with GST or GST-PRL-1 C104S fusion proteins (1 μg), or with the indicated PRL-1 protein fragments attached to glutathione-Sepharose beads in 500 μl of binding buffer (20 mM HEPES, pH 7.5, 150 mM NaCl) for 1 h at 4 °C with gentle rotation. The beads were washed five times with binding buffer and resuspended in Laemmli sample buffer, and the sample was analyzed by SDS-PAGE followed by autoradiography.

**Com-immunoprecipitations—** The two proteins or protein fragments being tested were translated and incubated with GST or GST-PRL-1 C104S fusion proteins (5 μl) and these were then sequenced. Sequence analysis was performed by electroporation. False positive results were eliminated by transforming the rescued plasmids back into the yeast strain HF7c.

**In Vitro Transcription/Translation—** In vitro transcription/translation was performed using the Tnt-coupled lysate system (Promega) according to the manufacturer’s instructions. The reaction was incubated for 2 h at 30 °C in the presence of [35S]methionine, and the products were analyzed directly by SDS-PAGE or subjected to immunoprecipitation or binding assays prior to SDS-PAGE as described in the text.

**GST Binding Assays—** Radiolabeled in vitro translated proteins (5 μl) were incubated with GST or GST-PRL-1 C104S fusion proteins (1 μg), or with the indicated PRL-1 protein fragments attached to glutathione-Sepharose beads in 500 μl of binding buffer (20 mM HEPES, pH 7.5, 150 mM NaCl) for 1 h at 4 °C with gentle rotation. The beads were washed five times with binding buffer and resuspended in Laemmli sample buffer, and the sample was analyzed by SDS-PAGE followed by autoradiography.

**Electrophoretic Mobility Shift Assays—** Preannealed, gel-purified, double-stranded oligonucleotides were radiolabeled and incubated with 5 μl of in vitro translated proteins or 10 μg of mouse liver nuclear extract for 15 min at room temperature in binding buffer (10 mM Tris, pH 7.5/50 mM NaCl/1 mM EDTA/1 mM dithiothreitol/5 mM MgCl2/10% (v/v) glyceraldehyde-3-phosphate dehydrogenase. The yeast strain HY7c was co-
Gal-4 Domain Fusion Constructs

| DNA-Binding       | Activating        | Growth on trp-leu- | Growth on trp-leu-his- | β-galactosidase filter assay |
|-------------------|-------------------|-------------------|-----------------------|-----------------------------|
| pGBT9-PRL-1-C104S-1-132 | pGAD                | +                 | -                     | -                           |
| pGBT9-PRL-1-C104S-1-132 | pGAD-ATF-7          | +                 | -                     | +                           |
| pGBT9 vector (BD only) | pGAD-ATF-7         | +                 | -                     | -                           |
| pVA3 (BD-p53 fusion) | pGAD-ATF-7         | +                 | -                     | -                           |

FIG. 1. ATF-7 interaction with PRL-1 in the yeast two-hybrid system. A two-hybrid screen was performed as described under “Experimental Procedures.” Of the 31 true positive clones identified, 16 encoded ATF-7. Results of the two-hybrid screen show that the interaction between the PRL-1-C104S-(1–132)-Gal4 DNA-binding domain and ATF-7-Gal4 activation domain fusions is dependent on the presence of both constructs and does not occur if empty vector controls or an irrelevant bait construct (p53) is used.

The sequence encodes a protein of 217 amino acid residues, with a predicted molecular mass of 24 kDa and a pI of 5.46. In vitro translation of the ATF cDNA yielded an ~30-kDa protein (Fig. 4), probably due to post-translational modification of the protein.

Comparison with data bases revealed that the sequence is novel but that it has several characteristics of a bZIP transcription factor. The extreme C-terminal end of the predicted ATF-7 protein contains three leucines and three valines, each separated by six other amino acids, suggesting a leucine zipper structure (38). This hybrid leucine-valine zipper is unique to ATF-7 among the previously described bZIP proteins. Immediately upstream of this leucine-valine zipper sequence is an arginine-lysine-rich basic domain, thought to be necessary for sequence-specific DNA binding by bZIP proteins (39, 40). The N-terminal end of the predicted protein is negatively charged and proline-rich, reminiscent of the acidic activation domains of bZIP transcription factors. The bZIP family can be divided into three groups on the basis of binding site preference (41): (i) the CREB/ATF family, which contains the ATF proteins, the original CREB proteins, and the CRE modulators. Distinctions within the bZIP family are also based upon differences in transactivating ability, patterns of tissue expression, and phosphorylation by specific kinases (41). As shown in Fig. 2B and summarized in Fig. 2C, ATF-7 is most closely related to ATF-4 (also known as CREB-2, C/ATF, and TATAF67 (42–45)). In a number of cases, especially near the C terminus, ATF-7 and ATF-4 contain identical amino acids that diverge from the consensus deduced from the other bZIP proteins (Tyr-227, Asp-230, Glu-234, Val-235, Lys-237, Arg-239, and Gln-241).

DNA Binding and Tissue Expression Pattern of ATF-7—Because bZIP proteins bind specific DNA elements, we next sought to confirm that ATF-7 is indeed a DNA-binding protein and identify which DNA sites ATF-7 can bind. We used electromobility shift assays to test the ability of in vitro translated ATF-7 to bind different DNA sequences known to bind by bZIP proteins. As shown in Fig. 2D, in vitro translated ATF-7 can bind as a homodimer to a CRE oligo probe (first lane). The specificity of this binding is underscored by the supershift of the DNA-protein complex by the addition of anti-ATF-7 antibody (second lane) and its elimination upon addition of an excess of cold competitor oligo (third lane). Conversely, the band is not eliminated by the addition of excess mutant oligonucleotide composed of the same nucleotides in a scrambled sequence (fourth lane). We have verified that the supershift shown in the second lane is due to ATF-7 and not a cross-reacting protein by obtaining the same results using Myc-tagged ATF-7 and anti-Myc tag (9E10) antibody (data not shown).

2 R. Diamond, unpublished data.
Because ATF-4 has been reported to bind to C/EBP sites (45–47), we also tested ATF-7's ability to bind to a C/EBP site oligo. These data are also shown in Fig. 2D. We found that ATF-7 could not bind this element as a homodimer. The single band that appears in the ATF-7 (second) lane, is also present when reticulocyte lysate alone is used (first lane). This band is not supershifted by the addition of anti-ATF-7 antibody (third lane) and is not eliminated by the addition of excess cold competitor oligo (fourth lane). The fifth lane shows results with liver nuclear extract. As expected, there is prominent binding evident, indicating that the failure of ATF-7 to bind is not due to a problem with the oligo or binding conditions. We have also shown). Because ATF-4 has been reported to bind to C/EBP sites (45–47), we also tested ATF-7's ability to bind to a C/EBP site oligo. These data are also shown in Fig. 2D. We found that ATF-7 could not bind this element as a homodimer. The single band that appears in the ATF-7 (second) lane, is also present when reticulocyte lysate alone is used (first lane). This band is not supershifted by the addition of anti-ATF-7 antibody (third lane) and is not eliminated by the addition of excess cold competitor oligo (fourth lane). The fifth lane shows results with liver nuclear extract. As expected, there is prominent binding evident, indicating that the failure of ATF-7 to bind is not due to a problem with the oligo or binding conditions. We have also

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**Fig. 2.** ATF-7 encodes a novel bZIP protein, most closely related to ATF-4, whose homodimers specifically bind to CRE elements. A, nucleotide and deduced amino acid sequence of the open reading frame of ATF-7. The bZIP domain is indicated by the solid line, the leucine-valine zipper residues are indicated in boldface, and the initiation (ATG) and stop (TAG) codons are boxed. B, comparison of the amino acid sequences of the bZIP domains of ATF-7 and several other bZIP proteins showing greatest degree of homology with ATF-4. Shaded areas indicate identical amino acids; boxed areas indicate homologous amino acids. In a number of cases, especially near the C terminus, ATF-7 and ATF-4 contain identical amino acids that diverge from the consensus deduced from the other bZIP proteins (see text for details). C, table summarizing the homology among the bZIP proteins shown in B. In D: left panel, CRE oligonucleotide was radiolabeled and incubated with in vitro translated ATF-7 (first lane) as described under “Experimental Procedures.” The mixtures were electrophoresed on a nondenaturing polyacrylamide gel, which was then dried and exposed to film. Supershift (second lane) and competition assays (third and fourth lanes) were performed as described in the text. Specific ATF-7 and nonspecific bands are indicated by arrows. Right panel, C/EBP oligonucleotide was radiolabeled and incubated with reticulocyte lysate alone (first lane), in vitro translated ATF-7 (second, third, and fourth lanes), or mouse liver nuclear extract (fifth and sixth lanes) as described under “Experimental Procedures.” The mixtures were processed as indicated for supershift and competition experiments in the same manner as was done for the CRE experiments. The supershift and cold competition data confirm that the band seen is a nonspecific band that is not due to ATF-7 binding.
not found evidence that ATF-7 homodimers can bind to AP1 sites (data not shown).

Northern blot analysis was performed to determine the tissue distribution of ATF-7, and, as shown in Fig. 3A, it was found to be expressed ubiquitously. The highest levels of expression appear to be in the liver, lung, adipose tissue, heart, and skeletal muscle. We also examined the expression pattern of ATF-7 in situations where PRL-1 has a distinctive pattern of expression. We did not find variation in the level of ATF-7 expression during liver regeneration (data not shown). We then examined the expression of ATF-7 in Caco-2 cells, a human colonic adenocarcinoma cell line, which exhibits spontaneous functional differentiation when the cells have grown to confluence. This differentiation is characterized by the development of an apical brush border, expression of high levels of intestine-specific enzymes such as lactase and sucrase, and the formation of a polarized cell layer with domes (48, 49). As shown in Fig. 3B, ATF-7 is expressed to a significantly greater degree in the post-confluent, differentiated cells than it is in the preconfluent undifferentiated cells. Interestingly, ATF-7's pattern of expression in these cells is reminiscent of that of PRL-1 (20).

**Confirmation and Mapping of the PRL-1-ATF-7 Interaction—Coimmunoprecipitation assays were performed to verify the interaction of ATF-7 with PRL-1 in vitro. As shown in Fig. 4A, anti-Myc-tag antibody (9E10) was able to coimmunoprecipitate in vitro translated ATF-7 along with Myc-tagged C104S-PRL-1 (lane 5), whereas control antisera did not (lane 4). The anti-Myc-tag antibody could not coimmunoprecipitate luciferase, a control in vitro translated protein along with Myc-tagged C104S-PRL-1 (lane 6), indicating that the coimmunoprecipitation of ATF-7 is specific.

In a similar manner, as shown in Fig. 4B, the anti-Myc-tag antibody was able to coimmunoprecipitate in vitro translated C104S-PRL-1 along with Myc-tagged ATF-7 (lane 5), whereas control antisera did not (lane 4). The specificity of this experiment was confirmed by demonstrating that the luciferase control protein could not be coimmunoprecipitated along with Myc-ATF-7 (lane 6). Taken together, these results confirm the interaction of PRL-1 and ATF-7 in vitro. To further confirm this interaction, a glutathione S-transferase (GST)-C104S-PRL-1 fusion protein was used in an in vitro binding assay with full-length in vitro translated ATF-7. As shown in Fig. 4C, the GST-C104S-PRL-1 protein bound to glutathione Sepharose beads interacts with full-length ATF-7 (lane 2), but ATF-7 did not interact with the control GST protein (lane 1). We also performed the same experiment using wild type GST-PRL-1 protein and obtained similar results (data not shown).

To determine the regions of PRL-1 that are important for the interaction with ATF-7, six truncated GST-PRL-1 constructs were synthesized and tested for their ability to bind in vitro translated ATF-7. The constructs made spanned different regions of the 189-amino acid full-length PRL-1. As shown in Fig. 5A, GST fused to PRL-1 amino acids 1–96, 60–118, or 118–173 are not able to bind to in vitro translated ATF-7, whereas GST constructs fused to PRL-1 amino acids 1–132 and 97–173 could bind to ATF-7. The results with the construct containing amino acids 1–132 are not surprising, because this construct corresponds to the "bait" construct used in the two-hybrid screen. Analysis of these data (see diagram in Fig. 5B) generated the hypothesis that the region comprising amino acids 97–132, which corresponds to PRL-1's PTPase domain, was critical in mediating PRL-1's ability to interact with ATF-7. Accordingly, we synthesized a GST fusion protein containing only amino acids 97–132, and determined, as shown in lane 1 of Fig. 5A, that it was able to bind in vitro translated ATF-7, albeit less efficiently than the aa 97–173 construct (lane 3 of Fig. 5A) or full-length PRL-1 (Fig. 4C). The region contained in the 97–132 construct spans the PRL-1's phosphatase domain and the 15 amino acids that follow it. Neither the phosphatase domain alone nor the 15-amino acid region alone is sufficient for ATF-7 binding, because neither the 60–118 nor 118–173 constructs, which contain the complete phosphatase domain or the 15-amino acid region, respectively, was able to bind ATF-7. In summary, these results indicate that the PRL-1 PTPase domain, combined with an adjacent small amino acid region, is necessary and sufficient for ATF-7 binding. In addition, there may be regions in the C-terminal of PRL-1 that contribute to ATF-7 binding, although they are not absolutely required.

To determine the regions of ATF-7 that are important for mediating the interaction with PRL-1, we employed GST bind-
Lanes 1–3 and visualized by autoradiography. Immunoprecipitation with an anti-Myc-tag antibody was then carried out as described under “Experimental Procedures,” and the products were resolved by SDS-PAGE and visualized by autoradiography. Lanes 1–3 show the results of the in vitro translation; lanes 4–6 show the results of the immunoprecipitation, which demonstrates that ATF-7 co-immunoprecipitates with the Myc-tagged C104S PRL-1 (full-length), Myc-tagged ATF-7, and luciferase control protein was performed in the presence of [35S]methionine as described under “Experimental Procedures.” Immunoprecipitation with an anti-Myc-tag antibody was then carried out as described under “Experimental Procedures,” and the products were resolved by SDS-PAGE and visualized by autoradiography. Lanes 1–3 show the results of the in vitro translation; lanes 4–6 show the results of the immunoprecipitation, which demonstrates that C104S PRL-1 co-immunoprecipitates with the Myc-tagged ATF-7. C, in vitro translated ATF-7 was incubated with GST or GST-PRL-1 C104S full-length fusion proteins attached to glutathione-Sepharose beads, and after washing, the sample was analyzed by SDS-PAGE followed by autoradiography. The bottom panel shows a Coomassie Blue-stained SDS-PAGE gel demonstrating that the fusion proteins were expressed and were of the expected size.

Ability of PRL-1 to Selectively Dephosphorylate ATF-7 in Vitro—Because our data indicated that the PRL-1 phosphatase domain was necessary for interaction with ATF-7, we sought to determine whether PRL-1 is capable of dephosphorylating ATF-7 in vitro. We used c-Src kinase to phosphorylate GST-ATF-7 on tyrosine. This kinase was not able to phosphorylate GST alone (data not shown). We split the products of the kinase reaction into equal aliquots and then used each in a phosphatase assay using either PRL-1, C104S inactive PRL-1 (MLT), or buffer. Because all three phosphatase reactions derive from the same common kinase reaction, the ratio of labeled ATF-7 to labeled c-Abl must be constant among the three tubes before the addition of the phosphatase or control. This ratio would not be affected by uneven division of the kinase reaction or uneven loading of the gel, because there would be more or less of both proteins in the same proportion. No change in this ratio would be seen if PRL-1 nonspecifically dephosphorylated both c-Src and ATF-7, because the phosphorylation of both would decrease. The degree to which this ratio decreases thus reflects selective dephosphorylation of ATF-7 by PRL-1. A representative result is shown in Fig. 6A. We found that significantly less ATF-7 remained phosphorylated relative to c-Abl after treatment with PRL-1 than after treatment with the C104S-PRL-1 or buffer controls. This indicates that PRL-1 was able to selectively partially dephosphorylate the tyrosine-phosphorylated ATF-7. The results of four separate experiments were quantified by densitometry and are shown in Fig. 6B. PRL-1 was significantly (p < 0.01) more able to dephosphorylate the labeled ATF-7 than either C104S-PRL-1 or buffer alone control.

**DISCUSSION**

Using the yeast two-hybrid system, we have identified ATF-7 as a novel bZIP protein that interacts with the PRL-1 nuclear PTPase. The interaction of PRL-1 and ATF-7 has been confirmed using GST binding and coimmunoprecipitation assays, and the sites of interaction have been mapped to include PRL-1’s phosphatase domain and ATF-7’s bZIP domain.

An important issue is the role of ATF-7 and its partner PRL-1 in cellular differentiation. There are a number of items that support the existence of such a role. We have previously shown that PRL-1 expression is associated with differentiation (20). Recently, we have also found that PRL-1 is expressed both in the adult and during development in a number of differentiating epithelial tissues, including intestine, stomach, kidney, and lung (17). We have also found that PRL-1 is expressed in
3T3-L1 adipocytes in association with differentiation, and we identified the ATF-7 and PRL-1 interaction by two-hybrid screening of a 3T3-L1 adipocyte library. We have further shown here that ATF-7 is expressed to a much greater degree in post-confluent, differentiated Caco-2 cells than in preconfluent undifferentiated cells, a pattern reminiscent of that of PRL-1. All of these findings suggest that ATF-7 may play an important role in the development and maintenance of differentiating epithelial tissues.

Ultimately, experiments involving ectopic overexpression or ablation of ATF-7 in specific cells and tissues will be necessary to determine whether it has a direct role in modulating cellular differentiation. A potential mechanism might involve the ZIPK/DLK kinase (50). It is interesting to note that the highly homologous ATF-4 protein has been shown to interact with this protein, which in turn has been linked to both differentiation and apoptosis (51, 52). Concomitant roles in differentiation and apoptosis are plausible in the intestine, where enterocytes sequentially pass through proliferation, differentiation, and apoptosis phases during their life cycle (53). Agents that induce differentiation in several tissue models have also been shown capable of promoting apoptosis. One example is the ability of butyrate to sequentially induce these two processes in intestinal cells (54–56).

We show here that tyrosine-phosphorylated ATF-7 can be selectively dephosphorylated by PRL-1 in vitro. The control of transcription through the regulation of transcription factor phosphorylation is a well-established concept (1, 2, 5, 6). However, our results must be interpreted with caution. Although they are consistent with the possibility that PRL-1’s cellular role may involve dephosphorylating ATF-7, much more work will need to be done before this can be established. We have observed only partial dephosphorylation of the labeled ATF-7 in these experiments, a result that could indicate that proper reaction condi-
tions are not present, or that ATF-7 is phosphorylated at multiple sites, only some of which are dephosphorylated by PRL-1. However, it is also possible that the basis for the PRL-1-ATF-7 interaction is not that of a phosphatase-substrate interaction. Future studies will be geared toward analyzing whether this phenomenon occurs in vivo, mapping the specific residue(s) that are affected, and determining the transcriptional consequences of such a reaction. If ATF-7 itself is not a target of PRL-1, it may serve to bring PRL-1 into proximity with its true substrates. In this manner, PRL-1 could influence transcription by acting on transcriptional cofactors or elements of the basal transcription machinery. In some situations, kinases or phosphatases may bind transcription factors but influence transcription by acting on proteins other than the transcription factors themselves. A classic example of this phenomenon is the regulation of the NFκB transcription factor by phosphorylation of its sequestering inhibitor IκB, which leads to IκB’s degradation (7, 57). Another example is the nuclear tyrosine kinase c-Abl, which binds to p53 and increases its transactivating ability without phosphorylating it. It is thought that Abl may be able to execute this function by phosphorylating the C-terminal domain of RNA polymerase II, which is known to be extensively phosphorylated on tyrosine (14, 15). Alternatively, ATF-7 may have extra-transcriptional roles involving sequestration of specific proteins (e.g. ZIPK) with roles in the regulation of apoptosis or differentiation, as has been proposed for the highly homologous ATF-4 protein (51, 52, 58). PRL-1 might impact upon these processes by dephosphorylating ATF-7 itself, or by acting on the target proteins bound by ATF-7.

We have determined that ATF-7 homodimers bind to CRE sites, and we have not found evidence that ATF-7 homodimers can bind to AP1 or C/EBP sites. Interestingly however, the only other published data about ATF-7 is the description of a partial clone, comprising only ATF-7’s bZIP region, that was identified in a Far Western screen as interacting with the C/EBPα transcription factor (59). This suggests that ATF-7 can interact with C/EBP family members, which are known to play important roles in the differentiation and development of a number of tissues, including liver and intestine (60–63). One manner in which this

FIG. 6. Tyrosine-phosphorylated ATF-7 can be specifically dephosphorylated in vitro by PRL-1. A, GST-ATF-7 was expressed in bacteria, purified, and tyrosine-phosphorylated with 32P using c-Src as outlined under “Experimental Procedures.” After the kinase reaction was stopped, the beads were washed four times and split into three equal aliquots. Equal amounts of active or mutant PRL-1, or buffer (negative control), were added to each tube, which were then incubated for 60 min at 37 °C. The phosphatase reaction was terminated by the addition of equal volumes of 2× Laemmli buffer. The products were then boiled and run on an SDS-PAGE gel, which was then dried and exposed to x-ray film (Kodak). Because all three phosphatase reactions derive from the same common kinase reaction, the ratio of labeled ATF-7 to labeled c-Src must be constant among the three tubes before the addition of phosphatase or control. (This ratio would not be affected by uneven division of the kinase reaction or uneven loading of the gel, because there would be more or less of both proteins in the same proportion.) No change in this ratio would be seen if PRL-1 nonspecifically dephosphorylated both c-Src and ATF-7, because the phosphorylation of both would decrease. The degree to which this ratio decreases thus reflects selective dephosphorylation of ATF-7 by PRL-1. A representative result is shown in A. We found that significantly less ATF-7 remained phosphorylated relative to c-Src after treatment with PRL-1 than after treatment with the C104S-PRL-1 or buffer controls. This indicates that PRL-1 was able to selectively partially dephosphorylate the tyrosine-phosphorylated ATF-7. The results of four separate experiments were quantified by densitometry and are shown in B. PRL-1 was significantly (p < 0.01) more able to dephosphorylate the labeled ATF-7 than either C104S-PRL-1 or buffer alone control.
could occur is through the formation of ATF-7-C/EBP heterodimers. It is a hallmark of bZIP proteins that they extensively heterodimerize with each other, both within and outside of individual families. These interactions are not completely promiscuous but are instead restricted by a specific and complex “dimerization code” (64, 65). In some cases, cross-family heterodimers bind to the preferred site of one of the dimer members (66, 67), whereas in other cases, the heterodimers bind to novel composite sites (45–47, 68–70). In some cases, dimerization occurs only with members of other bZIP families. In this regard, it is interesting to note that ATF-4, the bZIP protein most similar to ATF-7, can form heterodimers with members of the C/EBP family, including C/EBPβ and C/EBPγ (45, 46, 68, 71), but has not been reported to heterodimerize with any other member of the ATF/CREB family. In these situations, the heterodimeric complexes bind to either C/EBP sites or to ATF-7/C/EBP composite sites (45–47, 70), although ATF-4 homodimers do not bind to either of these sites. In addition, ATF-7 has also been reported to form heterodimers with the AP1 proteins Fos and Jun (72). It is possible that ATF-7 may play an important role in transcriptional regulation in a similar manner as ATF-4 through interaction with C/EBP and/or AP1 family members. Future experiments will be designed to address this issue.

In summary, the identification of the ATF-7-PRL-1 interaction may not only provide information about how PRL-1 may bring about the phenotypic states with which it is associated but also may have important implications for our understanding of the transcriptional regulation of target genes that modulate differentiation.

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Note Added in Proof—While this manuscript was under review, sequences for mouse and human ATF-5 were deposited in GenBank<sup>TM</sup>. It appears that ATF-7 and ATF-5 are likely to be the same protein. In addition, an unrelated sequence named ATFP has also been deposited in GenBank<sup>TM</sup>. In order to avoid confusion, future work on the protein described in this publication will likely refer to it as either ATF-5 or ATF-7.

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