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A Two-Step Mechanism for Recruitment of Pip by PU.1

Jeffrey M. Perkel* and Michael L. Atchison2

Transcription of the Ig κ light chain gene is controlled in part by the 3′ κ enhancer. Two of the proteins that bind to the 3′ enhancer, PU.1 and Pip, show tissue-restricted expression and may be responsible for the tissue specificity of 3′ enhancer activity. PU.1 alone can bind to DNA; however, Pip cannot bind to its 3′ enhancer site in electrophoretic mobility shift assays, unless recruited by PU.1. Previously, we showed that the PU.1 PEST domain (rich in the amino acids proline, glutamate, serine, and threonine; sequences 118–160) is necessary for Pip recruitment to DNA. Here we used detailed mutagenic analyzes of PU.1 to more precisely identify sequences required for Pip recruitment by electrophoretic mobility shift assay. We found that mutation of three segments within the PU.1 PEST domain (118–125, 133–139, and 141–147) modulated the efficiency of Pip recruitment, while mutation of segments between residues 88–116 and 154–168 had no effect. Interestingly, we found that the PU.1 ETS domain (residues 170 to 235) is both necessary and sufficient for Pip interaction in solution and that other ETS domain proteins can physically interact with Pip as well. Our results suggest that Pip recruitment to DNA by PU.1 occurs via a two-step mechanism. First, a physical interaction that is not sufficient to recruit Pip occurs via the PU.1 ETS domain. Second, a conformational change in Pip enabling it to bind to DNA. We also show that the PU.1 PEST domain does not target PU.1 for rapid turnover. The Journal of Immunology, 1998, 160: 241–252.

Several structural features of the PU.1 protein have been identified. The amino terminus of the protein contains the transcriptional activation domain, which includes both acidic and glutamine-rich domains (25). A PEST domain (rich in the amino acids proline, glutamate, serine, and threonine) is located between amino acids 120 and 160, and the DNA-binding ETS domain is located between amino acids 161 and 260 (9). Although PU.1 is capable of binding to its cognate DNA site by itself, we previously identified a nuclear factor, NF-EM5, that is incapable of binding to the Igκ 3′ enhancer unless it is recruited by PU.1 (6, 17). The PU.1 and NF-EM5 DNA-binding sites lie adjacent to one another within the 3′ enhancer. A similar arrangement of PU.1 and NF-EM5 binding sites occurs within the Igκ enhancer (17). PU.1 sequences necessary for recruitment of NF-EM5 to DNA when assayed by electrophoretic mobility shift assay (EMSA) include amino acids 118–160 (the PEST domain; Ref. 6). In addition, recruitment is critically dependent upon phosphorylation of serine 148 in the PU.1 PEST domain (16).

Recently, a protein with properties strongly matching those of NF-EM5 was cloned (26–28). This protein, named Pip (variously called LSIFR, ICSAT, or IRF4), is related to the IFN-regulatory factors (IRFs) and is a lymphoid-restricted transcription factor. Pip function is critical for proper immune function because disruption of the Pip gene by homologous recombination causes a deficiency in B cell Ag response and in cytotoxic and antitumor T cell activity (29). Interestingly, Pip can bind, in the absence of PU.1, to the IFN-stimulated response element (ISRE (27, 28)). However, full length Pip is unable to bind to its DNA-binding site in the 3′ κ and λ enhancers when assayed by EMSA (26). As expected, incubation in the presence of PU.1 results in recruitment of full length Pip to these DNA-binding sites. Pip C-terminal sequences appear to mask the Pip DNA-binding domain, thus inhibiting its ability to bind to κ and λ enhancer sequences (30). Pip interaction with PU.1 may

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Abbreviations used in this paper: PEST, domain rich in the amino acids proline, glutamate, serine, and threonine; NETN, 100 mM NaCl/1 mM EDTA/20 mM Tris, pH 8.0/0.5% Nonidet P-40; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase.

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therefore result in a conformational change in Pip such that its DNA-binding domain becomes unmasked (30). However, the precise mechanism by which Pip is recruited to DNA by PU.1 remains unknown.

In this report, we have used mutagenesis to better define the region of PU.1 required for recruitment of Pip to its Ets sites in tandem with the reverse primers in Table I to amplify the 3’ portion of PU.1. The PU.1 mutants, 7–30, 33–100, 154–160, and 156–164, plus the alanine substitution mutants were generated using 30 ng of template (25.1), 300 ng of each primer, 50 mM each of dATP, dCTP, dGTP, and dTTP, 1× PCR buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.001% (w/v) gelatin (Perkin-Elmer/Cetus, Norwalk, CT), 2.5 U of AmpliTaq polymerase (Perkin-Elmer/Cetus), and 2.5 U of AmpliTaq Extender (Stratagene). The amplification cycles consisted of 35 cycles of denaturation for a 5 min (95°C), annealing for 30 s (50°C), and polymerization for 2 min (72°C), followed by 1 cycle of polymerization for 4 min (72°C).

The T3 primer (AATTAACCCCTCACAATTTAAGGG) was used in tandem with the reverse primers in Table I to amplify the 5′ portion of PU.1, while the primer PU.1 3′UT Rev (GGCGTCTAGCGTCTCGTGGGCGGGCAAGG) was used in tandem with the forward primers in Table I to amplify the 3′ portion of PU.1. The PU.1 mutants, 0.9 kb EcoRI-BglI fragment from the plasmid 25.1 into Smal-digested pKS1. The Ets-1 cDNA was provided by Dr. Barbara Graves (University of Utah, Salt Lake City, UT), the Fli-1 cDNA was provided by Dr. Rich Maki (La Jolla Cancer Research Foundation, La Jolla, CA), and the Ets-2 cDNA was provided by Dr. Narayan Avadhani (University of Pennsylvania, Philadelphia, PA).

PU.1 deletion mutations were generated by PCR amplification of regions of the PU.1 cDNA clone 25.1 (9) that flank the desired deletion, followed by digestion with appropriate enzymes and ligation of the two pieces into pKS1-Bluescript (Stratagene, La Jolla, CA). The PCR reactions were conducted using 30 ng of template (25.1), 300 ng of each primer, 50 mM each of dATP, dCTP, dGTP, and dTTP, 1× PCR buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.001% (w/v) gelatin (Perkin-Elmer/Cetus, Norwalk, CT), 2.5 U of AmpliTaq polymerase (Perkin-Elmer/Cetus), and 2.5 U of Tag Extender (Stratagene). The amplification cycles consisted of 35 cycles of denaturation for a 1 min (95°C), annealing for 30 s (50°C), and polymerization for 2 min (72°C), followed by 1 cycle of polymerization for 4 min (72°C).

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Electrophoretic mobility shift assays

Nuclear extracts were prepared from S194 plasmacytoma cells essentially as described (32), and binding reactions were performed as previously described (6). The sequence of the PU.1-Pip-binding site from the Igk 9 enhancer used as a probe in these assays is CTTTGAGGAACCTGAAA CAGAACCT (Oligo 5). Quantification of EMSA data was conducted by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). For determination of off-rates, binding reactions were conducted until PU.1:Pip:DNA complex formation reached equilibrium (20 min) followed by addition of a large excess of cold competitor oligonucleotide. Aliquots were removed at various time points and loaded onto a nondenaturing gel. After electrophoresis, the fraction of probe retained in the free DNA, PU.1:DNA, and PU.1:Pip:DNA complexes were quantified using PhosphorImager analysis. The percentage of probe in the PU.1:Pip:DNA complex at a given time point (i.e., the percent of counts in the complex based upon the total number of counts in all three complexes) was normalized based upon a value of 100% for no competitor added.

Proteasome sensitivity studies

Conformational differences between selected PU.1 mutants were studied by partial proteolytic digestion of PU.1:DNA complexes that had formed in standard EMSA reactions, according to the method of Lodie et al. (33). After the reactions had reached equilibrium, 1 to 2 μl of solution containing either trypsin or proteinase K was added, and the reactions were allowed to proceed for 5 min at room temperature. The reactions were then stopped on ice and subjected to electrophoresis under standard EMSA conditions.

Glutathione S-transferase (GST) chromatography experiments

GST-fusion proteins were prepared essentially as described (34). Equivalent TCA-precipitable counts of [35S]-labeled rabbit reticuloocyte lysate translated proteins (Promega, Madison, WI) or metabolically labeled proteins (see below) were incubated with approximately equivalent amounts (as judged by Coomasie blue staining) of GST or GST-fusion proteins bound to glutathione-agarose beads for 1 h at 4°C in NETN (100 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 0.5% Nonidet P-40). The beads were washed four to five times in NETN, and bound proteins were eluted in 1× SDS loading dye and resolved on 10% SDS polyacrylamide gels. When oligonucleotides were included in the assay, 500 ng of oligonucleotide was added. The following oligonucleotides were used: AGCAACT GTCATAGCTACCGTCACA (nonspecific (NS)), CTTTGAGGAACT GTCATAGCTACCGTCACA (nonspecific (NS)), CTTTGAGGAACT GTCATAGCTACCGTCACA (nonspecific (NS)), CTTTGAGGAACT GTCATAGCTACCGTCACA (nonspecific (NS)), CTTTGAGGAACT GTCATAGCTACCGTCACA (nonspecific (NS)), CTTTGAGGAACT GTCATAGCTACCGTCACA (nonspecific (NS)).

Metabolic labeling and immunoprecipitation experiments

For metabolic labeling, a variation of the method of Luscher and Eisenman was used (35). Briefly, 3T3 fibroblasts were transfected via the CaPO4 precipitation method (36) with 10 μg of either plasmid CMV-PU.1 or CMV-PU.1PEST, or both. Twenty-four hours later, cells were pulsed with 0.2 mM/cml [35S]-protein-labeling mix (Express[35S][35S]; Dupont-NEN, Boston, MA) for 2 h at 37°C followed by a chase with cold methionine (0.5 mM). At the specified time points, the cells were harvested in Ab buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 0.5% Nonidet P-40, 0.5% SDS, 0.5% deoxycholate, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml N-a-p-tosyl-L-arginine methyl ester, 1 μg/ml pepstatin, 1 mM PMSF), sonicated, and clarified by centrifugation. Approximately 1.5 × 106 TCA-precipitable counts of each lysate was used in precipitations with excess anti-PU.1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA), and the immune complexes were recovered using goat anti-rabbit IgG-coupled agarose beads. The immune complexes were eluted by addition of SDS loading dye, resolved on SDS-polyacrylamide gels, and visualized by autoradiography.

For preparation of metabolically labeled lysates used in GST-pull down experiments, 3T3 fibroblasts were transfected with 20 μg of the PU.1 or PU.1PEST-expressing plasmids and labeled essentially as described above. Cells were harvested in hypotonic buffer (25 mM Tris-HCl, pH 7.4, 1 mM MgCl2, 5 mM KCl) and incubated on ice for 5 min. An equal volume of hypotonic buffer containing 1% Nonidet P-40 was added, and incubation was continued for an additional 5 min. The nuclei were pelleted and resuspended in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 8.0). The cell suspension was sonicated and clarified by centrifugation.

Results

NF-EM5 activity in S194 nuclear extracts is due to Pip

Brass et al. (30) recently showed that Pip is responsible for NF-EM5 activity in J558L cells. To determine whether the NF-EM5 activity present in S194 plasmacytoma cell nuclear extracts is also due to Pip, we performed EMSA studies with anti-Pip antisera. The PU.1:Pip DNA-binding sequence from the Igk 9 enhancer was incubated with S194 nuclear extract and either preimmune sera or anti-Pip sera. The PU.1:Pip:DNA complex was completely abolished by inclusion of a polyclonal Pip antiserum, while preimmune serum had no effect (Fig. 1). Therefore, the Pip protein present in S194 cells is identical, or highly homologous, to recombinant Pip. Because we obtained more efficient DNA binding with proteins isolated from nuclear extracts, the remainder of our studies were performed with Pip derived from S194 cells.

Identification of PU.1 PEST domain sequences necessary for Pip recruitment

We previously showed that PU.1 sequences 118–160 (the PEST domain) and phosphorylation of serine 148 are necessary for Pip recruitment to DNA by EMSA (6, 16). In addition, mutation of PU.1 serines 117, 132, or 133 to alanine residues reduced, but did not abolish, Pip recruitment (16). To better characterize the PU.1 PEST domain sequences important for Pip recruitment, we prepared a number of PU.1 PEST domain mutants using a PCR-based technique. Fifteen internal deletions were generated that had deletions anchored on the N-terminal side at arginine residues 87, 117, or 140 and on the C-terminal side at serine residues 107, 113, 126, 133, 142, 148, or 168 (Fig. 2A). Each mutant was transcribed and translated in vitro using [35S]methionine and assayed by EMSA for the ability to recruit Pip from S194 nuclear extracts to the PU.1:Pip DNA-binding site of the Igk 9 enhancer (sequences 445–469; numbering according to Ref. 3). The results of these assays are shown in Figure 2B.

Deletion of PU.1 amino acids 88–108 or 88–112 had no effect on the ability of PU.1 to recruit Pip to the DNA (Fig. 2B, lanes 1 and 2). Deletions of amino acids 118–122 and 118–132 or 128–132 reduced Pip recruitment, indicating that the amino acids in this region are important for Pip recruitment to DNA. Deletions of amino acids 133–142 reduced Pip recruitment to the DNA, while deletions of amino acids 143–162 reduced Pip recruitment to the DNA (Fig. 2B, lanes 3 and 4). Deletion of amino acids 143–162 abolished Pip recruitment to the DNA (Fig. 2B, lanes 5 and 6). These results indicate that Pip recruitment to the DNA depends on the presence of amino acids 133–162 in the PEST domain.

To better characterize the deletion mutants, we determined the ability of Pip to recruit Pip to the DNA in the presence of increasing concentrations of Pip antisera. The results of these experiments are shown in Figure 2C. The plot of antisera concentrations versus the ratio of Pip recruitment to DNA for the deletion mutants is shown in Figure 2C. The plot of antisera concentrations versus the ratio of Pip recruitment to DNA for the deletion mutants is shown in Figure 2C. The plot of antisera concentrations versus the ratio of Pip recruitment to DNA for the deletion mutants is shown in Figure 2C. The plot of antisera concentrations versus the ratio of Pip recruitment to DNA for the deletion mutants is shown in Figure 2C.
FIGURE 2. Identification of PU.1 PEST domain sequences important for Pip recruitment to DNA. A, Map of PU.1 mutants and their relative ability to recruit Pip. Each mutant PU.1 protein is diagrammed with the deleted or alanine substituted PU.1 sequences indicated on the left. The positions of the activation, PEST, and ETS domains are indicated. The shaded box represents the PEST domain. To the right is shown the relative ability of each mutant to recruit Pip to its DNA-binding site. ++ +, Indicates binding as efficient as wild-type PU.1; +, the ratio of the PU.1:Pip complex to the PU.1 complex is about 1:1; +, the PU.1 complex is more intense than the PU.1:Pip complex. B, EMSA with the kappa 3 enhancer PU.1-Pip DNA-binding site probe, the various PU.1 mutants prepared by in vitro transcription and translation, and Pip derived from S194 plasmacytoma cell nuclear extracts. The identity of each mutant PU.1 protein is indicated above each lane. The positions of the PU.1:Pip:DNA complex, PU.1:DNA complex, and free DNA probe are indicated by arrows.
Additional deletion to amino acid 125 or 132 resulted in a decrease in Pip recruitment ability (lanes 4–5). Similarly, deletion of amino acids 118–125 or 118–132 (lanes 9–10) resulted in a decrease in Pip binding. Deletion of amino acids 141–147 or 141–167 resulted in complete loss of Pip recruitment (lanes 6–8). Similarly, deletion of amino acids 118–125 or 118–132 (lanes 9–10) resulted in a decrease in Pip binding. Deletion of amino acids 141–147 or 141–167 completely eliminated Pip recruitment (lanes 11–13). Therefore, PU.1 sequences between 118 and 141 are necessary for Pip recruitment. Deletion of amino acids 141–147 or 141–167 also resulted in complete loss of Pip recruitment (lanes 15–16). Interestingly, deletion of a single amino acid, glutamine 141, produced a PU.1 mutant that appears to recruit Pip with higher efficiency than wild-type PU.1 (lane 14). Similar results with each mutant were seen using recombinant Pip protein (data not shown).

**Definition of the N-terminal and C-terminal limits of the recruitment domain**

The above results suggested that amino acids 113–125 contain the N-terminal boundary of the Pip recruitment domain (compare Δ88–112 to Δ88–125; Fig. 2B, lanes 3–4). We made two additional deletions to more precisely determine the N-terminal recruitment boundary (Δ111–114 and Δ111–117; Fig. 2A) and assayed them for Pip recruitment via EMSA. Neither deletion resulted in any loss of Pip recruitment relative to wild-type PU.1, indicating that sequences amino terminal to residue 118 are not required for Pip recruitment to DNA (Fig. 3A, lanes 1–3). Therefore, the N-terminal boundary of the Pip recruitment domain lies between PU.1 amino acids 118 and 125.

To define the C-terminal limit of the Pip recruitment domain, we made three small PU.1 deletions between amino acids 154 and 168 (Δ154–156, Δ161–164, and Δ165–168; Fig. 2A). None of these mutations caused any significant loss of Pip recruitment in EMSA (Fig. 3A, lanes 10–12), suggesting that PU.1 sequences between residues 154 and 168 are not necessary for Pip recruitment to DNA. Since mutation of serine 148 abolishes Pip recruitment (16), the C-terminal boundary of the Pip recruitment domain lies between the PU.1 residues 148 and 154.

**Subregions within the PEST domain**

Because deletion of amino acids 118–132 reduced Pip recruitment, whereas deletion of sequences 118–141 abolished recruitment, important sequences must also reside between amino acids 132 and 141. To better characterize these sequences, we made three additional deletions between amino acids 129–141: Δ129–132, Δ133–139, and Δ129–141 (Fig. 2A). Deletion of amino acids 129–132 had no effect on Pip recruitment compared with wild-type PU.1 (Fig. 3A, compare lanes 6 and 7). However, deletion of amino acids 133–139 resulted in a decrease in Pip recruitment, as did deletion of amino acids 129–141 (lanes 8 and 9).

Our results indicate that three PU.1 regions (sequences 118–125, 133–139, and 141–147) can influence Pip recruitment when deleted. However, deletion can potentially affect function by altering protein structure nonspecifically. Therefore, to better characterize these regions, we also prepared alanine point mutations between residues 118–125, 126–132, 133–139, and 141–147 (Fig. 3C).
Consistent with our deletion data, alanine mutants 118–125A and 133–139A reduced Pip recruitment to DNA, whereas mutant 126–132A had no effect (Fig. 3B, lanes 1–4). Alamine mutant 141–147A reduced Pip recruitment (Fig. 3B, lane 5), whereas deletion of the same sequences abolished recruitment (Fig. 2B, lane 15). This difference may be the result of lost phosphorylation of serine 148 in the deletion mutant (discussed below). Quantification of the recruitment efficiency of each alanine mutant as compared with wild-type PU.1 is shown in Figure 3C. Mutation of residues 118–125A was the most severe, reducing Pip recruitment >15-fold, while 133–139A and 141–147A reduced recruitment 2.5-fold and 9-fold, respectively.

Figure 2A contains a summary of each PU.1 mutant and Figure 4 shows the PU.1 PEST sequence and the specific sequences that influence Pip recruitment. Deletion or alanine point mutation of residues 118–125 (subregion A) or 133–139 (subregion B) reduced Pip recruitment, indicating that these sequences can modulate recruitment efficiency. Deletion of both regions (Δ118–141) completely eliminated Pip recruitment to the DNA, whereas mutation of the sequences between subregions A and B had no effect on Pip recruitment. Finally, alanine point mutation of residues 141–147 (subregion C) reduced Pip recruitment, whereas deletion of these sequences abolished recruitment.

### Mutants that reduce Pip recruitment have altered DNA-protein kinetics

Since deletion of sequences between PU.1 residues 118–125 and 133–139 modulated the efficiency of Pip recruitment, we sought to determine whether these mutants affected protein-DNA binding kinetics. To test this hypothesis, Δ118–125, Δ133–139, and wild-type PU.1 proteins were assayed by off-rate EMSA experiments. These studies showed differences in the off-rates of the PU.1:Pip DNA complex when comparing wild-type PU.1 with the mutant proteins (Fig. 5A). Interestingly, these studies showed a slower off-rate by the Δ133–139 mutant as compared with wild-type PU.1. The off-rate for the Δ118–125 mutant was intermediate between wild-type PU.1 and the Δ133–139 mutant. A similar trend was observed with the PU.1:DNA complex alone (data not shown). The different off-rates of the Δ118–125 and Δ133–139 mutants compared with wild-type PU.1 suggested that these proteins might have altered conformations. To determine whether this was the case, we performed protease sensitivity studies. Wild-type PU.1, Δ118–125, and Δ133–139 proteins were bound to DNA, then subjected to increasing doses of either trypsin (Fig. 5B) or proteinase K (Fig. 5C). Interestingly, both mutant proteins were slightly more resistant to protease digestion than was wild-type PU.1; this can best be seen when comparing the lowest doses of trypsin (Fig. 5B, compare lanes 2, 6, and 10) or proteinase K (Fig. 5C, compare lanes 2, 6, and 10).

The studies detailed above indicate that the sequences deleted in the mutants Δ118–125 and Δ133–139 can influence the recruitment of Pip to DNA. Mutation of these sequences can influence the kinetics of DNA-protein complexes and can alter the conformation of these proteins. It is interesting that the Δ133–139 mutant recruits Pip less efficiently than the wild-type protein, yet has a slower off-rate with Pip compared with wild-type PU.1. This issue will be addressed in the Discussion below.

**Interaction of GST-Pip and PU.1 in solution requires the PU.1 ETS domain**

The studies described above identified PU.1 sequences within the PEST domain that are required for efficient recruitment of Pip to its DNA-binding site as measured by EMSA. These studies require intact DNA-binding domains because mutation of the PU.1 ETS domain abolishes Pip recruitment (6). To study the interaction between PU.1 and Pip by a second method, we prepared a glutathione S-transferase-Pip fusion protein (GST-Pip) which enabled us to assay the PU.1-Pip interaction in solution. Bacterially produced GST-Pip protein was incubated with [35S]-labeled DNA, and wild-type PU.1 and Pip can physically interact in solution (Fig. 6A). Binding of wild-type PU.1 protein was easily detected in the absence of Pip, indicating that GST-Pip protein was used for the GST-Pip interaction (Fig. 6A, lanes 3–4). This unexpected result led us to test the specificity of this interaction under more stringent conditions. First, we washed the chromatography assays with NETN containing high salt and blocking protein (0.5 M NaCl, 0.5% milk). This treatment did not affect the relative binding of wild-type and mutant PU.1
proteins (data not shown). Second, we tested the relative specificity of the PU.1:PEST domain. A, PU.1 and PU.1Δ141–147 were prepared by in vitro transcription and translation in the presence of [35S]-labeled methionine. Equal numbers of counts of each protein were incubated with either GST, or GST-Pip. After incubation and washing, bound proteins were analyzed by SDS-PAGE. The first two lanes show 10% input of each protein. Above each lane is indicated the presence of either PU.1 or PU.1Δ141–147, and GST, or GST-Pip. Lanes received either no oligonucleotide (None), PU.1-Pip binding site sequences (PU.1:Pip), or a nonspecific oligonucleotide (NS). B, Interaction of PU.1 with GST-Pip is highly specific. NIH3T3 cells were transfected with either no plasmid (UnTx), CMV-PU.1 plasmid (PU.1), or CMV-PU.1ΔPEST plasmid (PU.1ΔPEST). Transfected cells were metabolically labeled, and nuclear extracts were prepared and subjected to chromatography with GST or GST-Pip beads. The 10% input of each lysate is shown in lanes 1, 4, or 7. Eluted proteins were subjected to SDS-PAGE. The arrows indicate the relative positions of PU.1 and PU.1ΔPEST.
FIGURE 7. The PU.1 ETS domain can physically interact with Pip. A and B, Various PU.1 mutants indicated above each lane were prepared by in vitro transcription and translation in the presence of [35S]-labeled methionine. Equal counts of each protein were incubated with either GST or GST-Pip. Bound proteins were eluted and subjected to SDS-PAGE. In B, 10% of each input protein is shown in the first three lanes. C, Summary of the GST chromatography experiments. The top rectangle represents wild-type PU.1, and gaps in the rectangles below represent sequences deleted in each mutant. The precise sequences deleted are indicated on the left. The relative ability of each mutant to interact with GST-Pip is shown on the right of each construct.
To identify the region of PU.1 that is required for interaction with GST-Pip, we tested a panel of PU.1 deletion mutants in GST chromatography assays. Proteins lacking the activation domain (D7–30 and D33–100; Fig. 7A, lanes 3–6), the PEST domain (D119–160; Fig. 7A, lanes 7–8), or both (D1–160; Fig. 7B, lanes 8–9) strongly interacted with GST-Pip. In contrast, PU.1 mutants D245–272 (Fig. 7A, lanes 9–10) and D201–272 (Fig. 7B, lanes 6–7), which lack portions of the ETS domain, were unable to bind to GST-Pip in these assays. These results are summarized in Figure 7C. Our results show that the PU.1 ETS domain is necessary and sufficient for physical interaction with GST-Pip. This domain, however, is not sufficient to recruit Pip to bind to DNA (6).

Pip interacts with some, but not all, ETS domain proteins

The observation that Pip can physically interact with the PU.1 ETS domain prompted us to determine whether other ETS domain proteins can interact with Pip. The ETS proteins Ets-1, Ets-2, and Fli-1 were tested. Results from these studies (Fig. 8) showed that Ets-1 interaction with GST-Pip was detectable but very weak (lanes 7 and 8), Fli-1 interacted with GST-Pip (lanes 11 and 12) although not as efficiently as PU.1 (lanes 5 and 6). Ets-2 was incapable of interacting with Pip (lanes 9 and 10). Therefore, some, but not all, ETS domain proteins can physically interact with GST-Pip.

The PU.1 PEST domain does not confer protein instability

Our results indicate a role of the PU.1 PEST domain in Pip recruitment to DNA. PEST domains have also been hypothesized to confer protein instability (37). We sought, therefore, to determine whether the PEST domain sequences play a role in PU.1 stability. NIH3T3 cells were transfected with plasmids expressing PU.1 or PU.1ΔPEST, or both, and 24 h post-transfection, cellular proteins were metabolically labeled with [35S]-labeled amino acids for 2 h, then chased with cold methionine for various time periods and harvested. The PU.1 proteins were immunoprecipitated from cell lysates with anti-PU.1 Ab and resolved by SDS-PAGE. As can be seen in Figure 9, the PU.1ΔPEST deletion is actually moderately less stable than the wild-type protein, indicating that the PEST domain does not confer an innate instability on PU.1.

Discussion

Role of the PU.1 PEST and ETS domains in interaction with Pip

To better characterize the PU.1 sequences important for Pip recruitment, we prepared 27 mutations that flank or traverse the PU.1 PEST domain. Consistent with our previous observations (6), we demonstrated that deletion or alanine point mutation of sequences within the PU.1 PEST domain significantly reduced or eliminated recruitment of Pip to DNA. Based upon the data summarized in Figures 24 and 4, multiple regions of the PU.1 PEST domain influence Pip recruitment. Mutation of residues 118–125 (subregion A), 133–139 (subregion B), or 141–147 (subregion C) reduced Pip recruitment.

The mechanism by which PU.1 sequences 118–125 (Met-Cys-Phe-Pro-Tyr-Gln-Thr-Leu) influence Pip recruitment is unknown. However, deletion of these sequences alters the conformation of PU.1, and this altered conformation may result in less efficient recruitment of Pip. This mutant protein might also serve as a less efficient substrate for phosphorylation of serine 148 by casein kinase (CK) II. However, addition of excess CKII did not increase...
with alanine residues. Therefore, the effects on Pip recruitment by mutation of residues 141–147 may be a consequence of reduced phosphorylation of serine 148. The multiple phosphorylated serines in PU.1 make it very difficult to determine the phosphorylation status of serine 148. This would require two-dimensional peptide mapping of metabolically labeled proteins harvested from transfected cells.

Somewhat surprisingly, mutants Δ118–125 and Δ133–139 showed slower off-rates from DNA as compared with wild-type complexes. Therefore, these mutants have a reduced ability to recruit Pip to DNA, but once assembled, the PU.1:Pip complex comes off the DNA more slowly. Possibly the mutant PU.1 proteins take on a conformation that stabilizes protein-DNA complexes, but they do not as readily undergo conformational changes that facilitate Pip recruitment (see below). Whether deletion of these sequences directly alters a sequence necessary for Pip recruitment or alters the structure of other PU.1 sequences necessary for this function is unclear.

We also studied the interaction of PU.1 and Pip in solution using GST chromatography. Surprisingly, in contrast to our EMSA data, deletion of the PU.1 PEST domain had no effect on PU.1:GST-Pip physical interaction. In fact, deletion of all PU.1 sequences except the ETS domain had no effect on PU.1:Pip interaction. On the other hand, deletions within the PU.1 ETS domain abolished the physical interaction between PU.1 and Pip. Therefore, the PU.1 ETS domain is necessary and sufficient for physical interaction with Pip. Since PU.1 belongs to a family of related ETS domain proteins, we tested the ability of several other ETS domain proteins to physically interact with GST-Pip. Interestingly, Fli-1 was also able to physically interact with GST-Pip, although the interaction between PU.1 and Pip appears to be more efficient (Fig. 8). Fli-1 and Pip are both found in B and T lymphocytes (26, 38, 39). It is therefore possible that Pip interacts with Fli-1 in either lymphocyte lineage. The ability of Pip to select from multiple potential dimerization partners may serve to modulate its activity by controlling DNA-binding site specificity, DNA-binding kinetics, or transactivation potential. It will be interesting to determine whether Pip normally interacts with other ETS domain proteins in addition to PU.1.

A two-step model for PU.1 recruitment of Pip to DNA

Two distinct PU.1 domains (the PEST and ETS domains) are required for the recruitment of Pip to its 3′ enhancer DNA site. Whereas both the PU.1 PEST and ETS domains are required for Pip recruitment to DNA, only the ETS domain is required for a solution interaction. This is supported by several observations. First, Pip recruitment via EMSA is eliminated by various deletions of the PEST domain, even though these clones contain the PU.1 ETS domain (Fig. 2A; see also Ref. 6). Second, the PU.1 ETS domain alone is incapable of recruiting Pip to its Igk 3′ enhancer DNA-binding site as assayed via EMSA (6). Third, mutations within the ETS domain abolish PU.1 DNA binding, and as a consequence, Pip recruitment (6). Finally, GST-Pip is able to interact with a variety of PU.1 mutants, including PU.1ΔPEST, but not with certain ETS domain mutants (Fig. 7).

A model consistent with the above observations is a modification of the one put forward by Brass et al. (30), who suggested that PU.1 interaction with Pip results in a Pip conformational change that exposes the Pip DNA-binding domain. Here, we propose a two-step mechanism that results in conformational changes in both PU.1 and Pip (Fig. 10). Initially, PU.1 can interact with Pip in solution via the PU.1 ETS domain. This interaction does not require the PU.1 PEST domain or PU.1 phosphorylation and does not result in recruitment of Pip to DNA. Second, phosphorylation...
of PU.1 at serine 148 causes a covalent structural change in the PU.1 protein. This structural change may directly induce changes in Pip (see below), or may induce a PU.1 conformational change perhaps involving PEST subregions A, B, and C (sequences 118–125, 133–139, and 141–147, respectively). Phosphorylation of PU.1 may occur either before or after interaction with Pip, but phosphorylation at serine 148 is critical because bacterial PU.1 is not capable of Pip recruitment unless first treated with CKII (16). The phosphorylation-induced change in PU.1 would then induce a subsequent conformational change in Pip that either exposes or alters the Pip DNA-binding domain, enabling recruitment of Pip to the \( \kappa^3 \) enhancer.

Evidence for a PU.1 conformational change is provided by the work of Lodie and coworkers (33), who showed that PU.1 in LPS-treated RAW264.7 macrophage cells are more resistant to proteolytic cleavage than PU.1 from unstimulated cells. This conformational change is accompanied by, and may be induced by, increased serine phosphorylation of PU.1. It is not presently known whether phosphorylation of PU.1 serine 148 is regulated during B cell development or in response to extracellular signals, but LPS treatment appears to be able to increase the phosphorylation state of this residue in RAW264.7 macrophages (33). It will be interesting to determine whether a similar mechanism is operative in B cells.

While the physical interaction between PU.1 and Pip is highly specific, other proteins are known to physically interact with PU.1. These include TATA-binding protein (TBP), the retinoblastoma protein (Rb), the glucocorticoid receptor (GR), NF-IL6\( \beta \), HMG I/Y, and structure-specific recognition protein (40–42). TBP, Rb, and GR appear to interact with the amino-terminal region of PU.1, whereas we show that Pip physically interacts with the carboxy-terminal PU.1 ETS domain (Fig. 7). Interestingly, NF-IL6\( \beta \) also physically interacts with the PU.1 ETS domain (42). However, the interaction between PU.1 and NF-IL6\( \beta \) does not result in cooperative DNA binding as is observed with PU.1 and Pip. The ability of the PU.1 ETS domain to physically interact with multiple proteins suggests that these interactions could be used as a regulatory mechanism. It will be interesting to determine whether NF-IL6\( \beta \) can disrupt the interaction between PU.1 and Pip.

PU.1 is a pivotal protein involved in hemopoietic development and in the genesis of erythroleukemia (10, 18–24). PU.1 can regulate expression of the human promyelocytic leukemia (H Ib) is dependent on promoter sequences which bind the hematopoietic transcription factor Sp1/PU.1. Mol. Cell. Biol. 15:58.


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