Physical and Functional Interactions between the Transactivation Domain of the Hematopoietic Transcription Factor NF-E2 and WW Domains†

Eric A. Mosser,‡ Jeremy D. Kasanov,‡ E. Camilla Forsberg,‡ Brian K. Kay,‡ Paul A. Ney,§ and Emery H. Bresnick*,‡

Department of Pharmacology, University of Wisconsin Medical School, 387 Medical Science Building, 1300 University Avenue, Madison, Wisconsin 53706, and Department of Biochemistry, St. Jude Children's Hospital, Room 4064, Thomas Tower, 332 North Lauderdale, Memphis, Tennessee 38105

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ABSTRACT: Tandem binding sites for the hematopoietic transcription factor NF-E2 in the β-globin locus control region activate high-level β-globin gene expression in transgenic mice. NF-E2 is a heterodimer consisting of a hematopoietic subunit p45 and a ubiquitous subunit p18. Gavva et al. [Gavva, N. R., Gavva, R., Ermekova, K., Sudol, M., and Shen, J. C. (1997) *J. Biol. Chem. 272*, 24105−24108] reported that human p45 contains a PPXY motif that binds WW domains. We show that murine NF-E2, which contains two PPXY motifs (PPXY-1 and -2) within its transactivation domain, differentially interacted with nine GST−WW domain fusion proteins. Quantitative analysis revealed high-affinity binding (K_D = 5.7 nM) of p45 to a WW domain from a novel human ubiquitin ligase homologue (WWP1) expressed in hematopoietic tissues. The amino-terminal WW domain of WWP1 formed a multimeric complex with DNA-bound NF-E2. A WWP1 ligand peptide, isolated by phage display, and a peptide spanning PPXY-1 inhibited p45 binding, whereas an SH3 domain-interacting peptide and a peptide spanning PPXY-2 did not. Mutation of PPXY-1, but not PPXY-2, inhibited the transactivation function of NF-E2, providing support for the hypothesis that WW domain interactions are important for NF-E2-mediated transactivation.

Long-range transcriptional regulation of the β-globin genes is mediated by a complex set of cis-acting elements termed the locus control region (LCR)[1] (reviewed in ref *1*). The LCR consists of four erythroid-specific DNase I hypersensitive sites (HS1−4) at the 5′-end of the β-globin locus (*2*−*4*). Each HS contains a variety of recognition sequences for erythroid-specific and ubiquitous transcription factors. Mutagenesis studies have shown that multiple factors are required for the activation property of the LCR (*5*). Of particular importance are conserved tandem binding sites for the erythroid- and megakaryocytic-specific transcription factor NF-E2 within HS2. Mutation of the NF-E2 sites almost completely abolishes activation by HS2 in transient and stable transfection assays as well as in transgenic mice (*5*−*8*). In contrast, mutations of other factor binding sites only partially inhibit the enhancer activity of HS2 (*5*), suggesting that these sites are less important than the NF-E2 sites.

NF-E2 is a heterodimeric b-zip transcription factor, consisting of a 45 kDa hematopoietic subunit (p45) (*9*, *10*) and an 18 kDa ubiquitous subunit (p18) (*11*). p45 is homologous to other b-zip proteins such as c-Fos and c-Jun, whereas p18 is a member of the Maf family of proteins (*12*), which are homologous to the v-Maf oncogene. The importance of NF-E2 in globin gene expression was shown in the mouse erythroleukemia cell line, CB3, which lacks p45 due to retroviral integration within the p45 locus (*13*, *14*). As CB3 cells do not express α- or β-globin genes, and globin gene expression can be rescued by forced expression of p45, p45 is critical for globin gene expression in this system.

Despite the p45 requirement for globin expression in CB3 cells, mice homozygous for a p45 gene deletion express globin genes and undergo erythropoiesis (*15*, *16*). A surprising finding from this knockout study was that p45 null mice lack platelets, indicating that p45 plays an essential role in megakaryopoiesis. However, the NF-E2 knockout mice did have a subtle microcytic anemia, indicating that globin synthesis was abnormal. In neonates, their blood smear was anemic, but one cannot exclude bleeding as a basis for this. This work suggested that redundant factors exist or that a feedback mechanism overrides the p45 requirement to ensure normal globin synthesis.

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* Corresponding author. Telephone: (608) 265-6446. Fax: (608) 262-1257. E-mail: ehbresni@facstaff.wisc.edu.

‡ University of Wisconsin Medical School.

§ St. Jude Children's Hospital.

[1] Abbreviations: b-zip, basic leucine-zipper transcription factor; BSA, bovine serum albumin; DTT, dithiothreitol; ELISA, enzyme-linked immunoassay; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase; HAT, histone acetylase; HECT, region homologous to the E6-AP carboxyl terminus; HS, hypersensitive site; LCR, locus control region; PBS, phosphate-buffered saline; PPXY, motif containing the PPXY sequence; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SH3, src homology domain 3; TAF, TATA binding protein-associated factor; YAP, Yes-associated protein.

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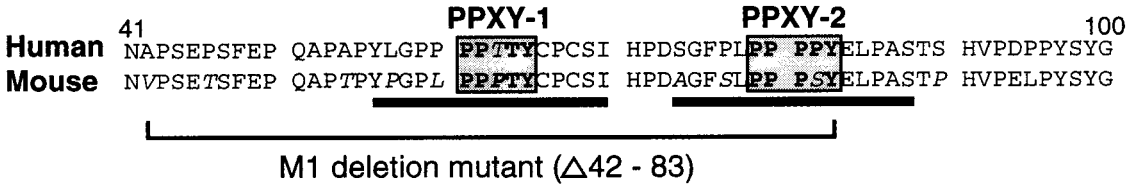

FIGURE 1: PPXY motifs of the p45 activation domain. The amino acid sequence of the amino-terminal region (residues 41−100) of human and mouse p45 is shown. Two PPXY motifs are shown in bold and by the shaded boxes. Amino acid residues of murine p45 that deviate from the human sequence are italic. An internal deletion mutant of p45 that was previously reported (*17*) to eliminate its transactivation activity (M1) is indicated by the bracket. The positions of peptides used in Figure 6 are indicated by the solid bars.

On the basis of the p45 requirement for megakaryopoiesis in mice and globin gene expression in CB3 cells, understanding how p45 activates transcription is of considerable interest. The amino acid sequences responsible for activation by p45 reside within its amino terminus (*17*). A deletion of amino acids 42−83 of murine p45 (M1 mutant) (Figure 1) strongly inhibits activation in the CB3 cell rescue assay. The interaction of p45 with three proteins has been suggested to be important for activation. First, a p45 derivative encompassing amino acids 1−269 has been shown to interact with a GST fusion protein containing amino acids 451−682 of the transcriptional coactivator CBP (*18*). CBP is a HAT (*19*) that mediates transcriptional activation by multiple transacting factors. Second, a GST fusion protein containing amino acids 1−80 of human p45 has been shown to interact with a GST fusion to the $TAF_{II}130$ protein (*20*). $TAF_{II}130$ is a component of the TFIID complex that assembles on core promoters. Third, the activation domain of p45 has also been shown to interact with protein modules called WW domains (*21*).

WW domains consist of 38−40 amino acids and bind proline-rich sequences with the consensus sequence PPXY (*22*−*24*). Under certain circumstances, the minimal motif PXY is sufficient for binding.[2] The activation domain of murine p45 contains one PPXY motif at amino acids 79−83 (PPPSY) that is conserved within human p45 (PPPPY). A second PPXY motif, at amino acids 61−65 (PPPTY) of the mouse sequence, is incompletely conserved in human p45 (PPTTY). Human p45 contains a third PPXY motif between amino acids 95 and 97, but this falls outside the functionally relevant region. Gavva et al. (*21*) showed recently that a GST−p45 fusion protein binds certain WW domains in a Far Western assay, and mutation of the PPXY motif at amino acids 80−83 abrogated binding. In this paper, we describe detailed studies on physical and functional interactions between the transactivation domain of p45 and WW domains.

EXPERIMENTAL PROCEDURES

Peptides. Peptides spanning amino acid sequences of the murine p45 amino terminus were synthesized by Research Genetics Inc. (Huntsville, AL). The mass and purity of the peptides were confirmed by mass spectroscopy analysis: PPXY-1 (amino acids 56−70), YPGPLPPPTYCPCSI; PPXY-1mt (amino acids 56−70 with PTY changed to AAA), YPGPLPPAAACPCSI; PPXY-2 (amino acids 74−88), AGFSLPPPSYELPAS; and Cont-1 (amino acids 21−35), LGEMELTWQEIMSIT. The lyophilized peptides were resuspended in 9% acetonitrile/10.2 mM NaOH. Peptides that interact with the Src SH3 domain (Src4, VLKRPLPIPPVTR) and the WWP1.1 WW domain (P1.1, SRGLPPPYDLTWVN) were isolated previously by phage display.[2] The amino terminus of the SH3 domain- and WW domain-interacting peptides contained a biotin residue followed by a SGSG or a KGSG linker, respectively.

Expression and Purification of Recombinant Proteins. Prokaryotic expression vectors containing the cDNAs for murine p45 and p18 were obtained from Beverly Emerson (Salk Institute) (*25*). Polyhistidine-tagged p45 and p18 were overexpressed in the BL21DE3pLysS and BL21DE3 strains of *Escherichia coli*, respectively. Bacterial cultures were grown to an A_{600} of 0.6, and then protein expression was induced with 1 mM IPTG for 2.5 h at 37 °C. Cells were isolated by centrifugation at 4220*g*. Proteins were purified on a column containing nickel−NTA resin equilibrated in buffer containing 8 M urea as described by the manufacturer (Qiagen). Protein samples were dialyzed overnight against 20 mM Tris, 0.1 mM EDTA, 5% glycerol, 50 mM NaCl, 5 mM DTT, and 0.5 mM PMSF at pH 7.8 and 4 °C with two changes of buffer. Protein concentrations were measured by Bradford assay using γ-globulin as a standard. The WW domains were amplifed by polymerase chain reaction from human cDNA clones (*26*) and cloned into the *Sal*I and *Not*I sites of the pGEX-4T-2 vector (Pharmacia) to generate GST fusion proteins.

Functional NF-E2 heterodimers were reconstituted by overexpressing p45 and p18 and purifying the proteins separately as described above. Equimolar amounts of purified p45 and p18 (2.3 nmol) were incubated in 0.5 mL of dialysis buffer containing 8 M urea, and the mixture was dialyzed overnight against dialysis buffer lacking urea. The reconstituted material was concentrated with a Centricon 10 microconcentrator to a final volume of 100 μL.

Electrophoretic Mobility Shift Assay. DNA binding was assayed by EMSA as described previously (*27*). Aliquots of recombinant NF-E2 heterodimers (1 μL) were incubated in 10 mM HEPES (pH 7.8), 60 mM KCl, 10% glycerol, 1 mM $MgCl_2$, 6 mM DTT, 2 μg of poly(dI-dC), 2 μg of BSA, and 40 fmol of end-labeled, double-stranded oligonucleotide duplex in a final volume of 20 μL for 20 min at room temperature. Certain reaction mixtures contained 1 μg of GST−WWP1.1, GST−NEDD4.2, or GST to determine whether the WW domains can form a quaternary complex with DNA-bound NF-E2; the GST−WW domain fusion proteins were added at the same time as other components. Samples were resolved on 6.5% nondenaturing polyacrylamide gels in 0.75× Tris-acetate/EDTA running buffer [30 mM Tris-acetate and 0.75 mM EDTA (pH 8.0)] at 200 V for 2 h at 4 °C.

[2] J. D. Kasanov, G. Pirozzi, A. J. Uveges, and B. K. Kay, manuscript in preparation.

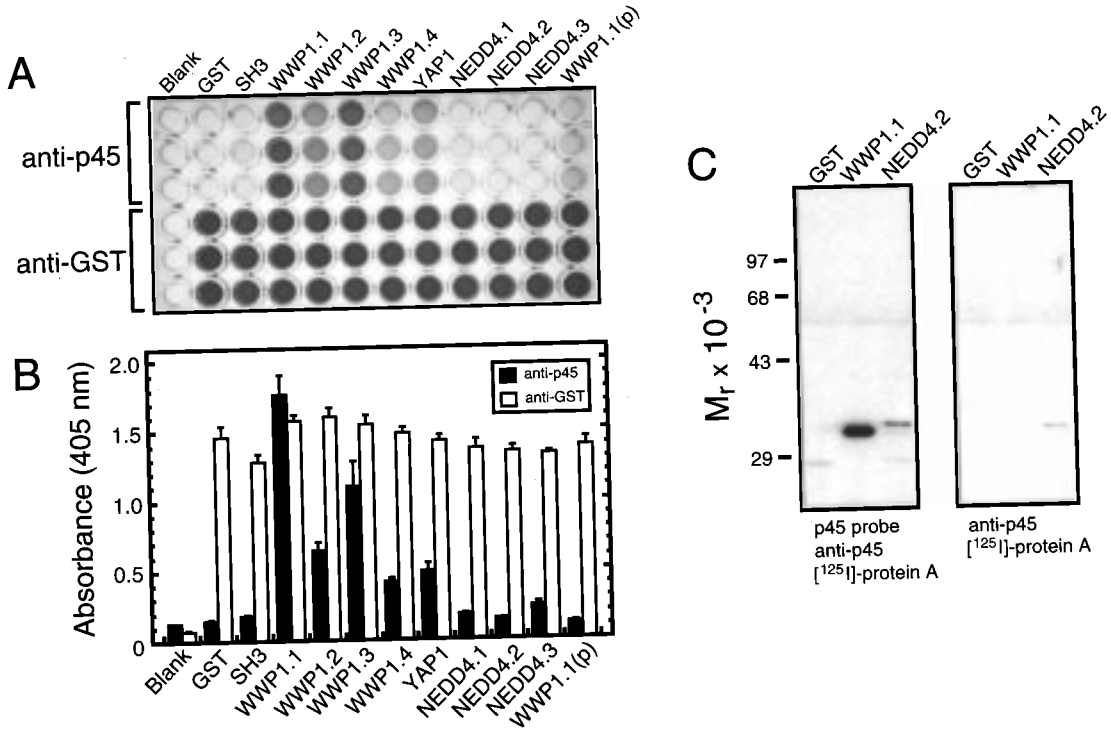

FIGURE 2: Differential binding of p45 to WW domains from WWP1, YAP65, and NEDD4 proteins. (A) GST and GST−SH3 and GST−WW domain fusion proteins were immobilized in wells of microtiter dishes. The immobilized proteins were incubated with purified p45, followed by anti-p45 antibody or anti-GST antibody, and then a peroxidase-conjugated secondary antibody. The antibody reactivity was visualized with a peroxidase substrate. Assays were performed in triplicate, with the top three rows incubated with anti-p45 antibody and the bottom three rows incubated with anti-GST antibody. WWP1.1(p) represents a control in which immobilized WWP1.1 was incubated with an unrelated nuclear protein (type II phosphatidylinositol phosphate kinase) followed by the anti-p45 antibody. (B) Quantitative analysis. The A_{405} values [mean ± standard error (SE), $n = 6$] were measured and plotted for the different conditions (solid bars, anti-p45; and open bars, anti-GST). (C) Far Western blot analysis of p45 binding to WWP1.1 and NEDD4.2. GST, GST−WWP1.1, and GST−NEDD4.2 were resolved by SDS−PAGE and analyzed by Far Western blotting using the p45 probe, followed by anti-p45 and [^{125}I]protein A. The control blot on the right was incubated with anti-p45 and [^{125}I]protein alone. The positions of molecular weight markers are indicated on the left.

ELISA of Protein−Protein Interaction. ELISAs were performed by a modification of the method described previously (*26*). Purified GST or GST fusion proteins (1 μg in the experiment shown in Figure 2 and 0.25 μg in the remaining experiments) were immobilized in triplicate wells of a 96-well ELISA plate (Costar EIA/RIA no. 3590) by incubation for 45 min in 100 μL of 0.1 M $NaHCO_3$. Nonspecific binding sites on the plastic were blocked by incubation with 100 μL of PBS containing 10 mg/mL BSA, 15 mM NaCl, and 0.001% Thimerosal for 1 h. Each well was washed three times with 300 μL of PBS/0.1% Tween 20. Immobilized proteins were incubated with 0.5 μg of purified p45 in 100 μL of PBS containing 0.1% Tween 20 (110 nM final concentration for p45) for 1.5 h. In Figure 3, the amount of p45 was varied. For peptide competition experiments, peptides were preincubated with immobilized proteins in 100 μL of PBS containing 0.1% Tween 20 for 30 min prior to incubation with p45. After five washes with 300 μL of PBS/0.1% Tween 20, the immobilized proteins were incubated with 50 μL of anti-p45 or anti-GST (Sigma) antibody solution (1/2000 and 1/4000 dilution of antisera in PBS/0.1% Tween 20 for p45 and GST, respectively) for 1 h. The rabbit anti-murine p45 antibody was produced against purified hexahistidine-tagged p45 and reacts specifically with human and mouse p45 by Western blotting, ELISA, and immunoprecipitation.[3] After five washes with 300 μL of PBS/0.1% Tween 20, the immobilized proteins were incubated with 50 μL of goat, anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Sigma) for 1 h. After five washes with 300 μL of PBS/0.1% Tween 20, hydrogen peroxide (0.06%) and the peroxidase substrate ABTS were added. Color development was allowed to proceed for 20 min. Absorbance measurements at 405 nm were taken with a Bio-Tek ELx800 plate reader under conditions in which the absorbance was in the linear range. All incubations were performed at room temperature with gentle agitation.

Far Western Protein−Protein Interaction Assay. Aliquots of recombinant proteins (0.5 μg) were boiled for 10 min in SDS−PAGE sample buffer and resolved on a 9% SDS−polyacrylamide gel. Proteins were transferred to Immobilon P membranes (Millipore) at 106 V for 1 h. Membranes were blocked by incubation in TBS buffer [25 mM Tris base, 13.7 mM NaCl, and 2.7 mM KCl (pH 7.4)] containing 0.1% Tween 20 and 10% nonfat dry milk for 12−14 h. Membranes were incubated with purified recombinant murine p45 protein (1.5 μg/mL) for 1.5 h in TBS/0.1% Tween 20/0.1% BSA. After three washes for 15 min each in TBS/0.15% Tween 20 buffer, membranes were incubated with rabbit anti-murine p45 polyclonal antibody (1/4000 dilution) in TBS/0.1% Tween 20/0.1% BSA for 1.5 h. After three washes for 15 min each in TBS/0.15% Tween 20, membranes were incubated with 0.25 μCi of [^{125}I]protein A (New England Nuclear) for 1.5 h. Membranes were washed three times with TBS/0.1% Tween 20, and immunoreactive bands were

[3] E. C. Forsberg, E. A. Mosser, A. Vassilev, and E. H. Bresnick, manuscript in preparation.

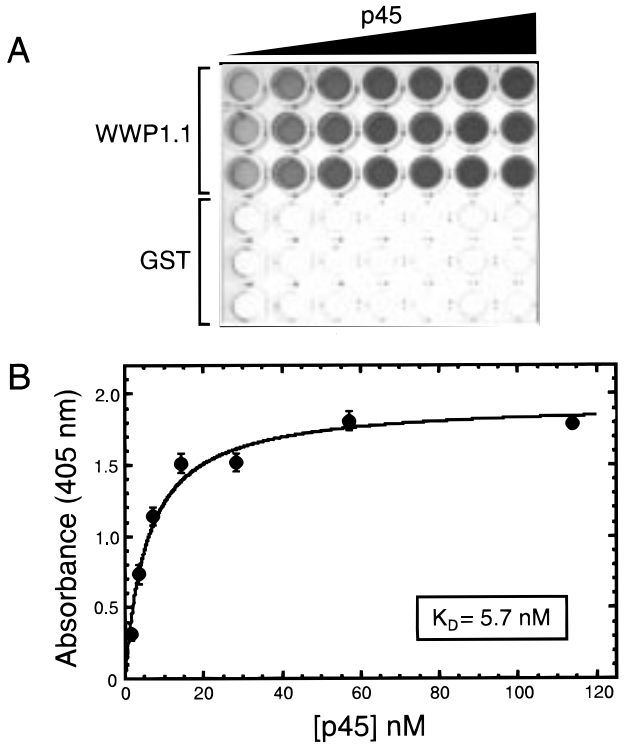

FIGURE 3: Quantitative analysis of p45 binding to WWP1.1. (A) ELISA assay. The binding of p45 at increasing concentrations to a constant amount of immobilized WWP1.1 was measured by ELISA. The GST−WWP1.1 fusion protein was immobilized in the top three rows of wells, and GST was immobilized in the bottom three rows. All wells were incubated with p45 followed by anti-p45 antibody and then a peroxidase-conjugated secondary antibody. Assays were performed in triplicate. (B) Quantitative analysis. A_{405} values for wells containing immobilized GST were subtracted from the corresponding values for wells containing immobilized WWP1.1 to yield specific binding values (mean ± SE, $n = 3$). Specific values were plotted against the concentration of the p45 probe. Nonlinear regression analysis was used to estimate the K_D for the p45−WWP1.1 interaction ($K_D = 5.7$ nM).

detected with a PhosphorImager. All incubations were performed at room temperature with agitation.

Northern Blot Analysis. Blots containing mRNA (2 μg) from human hematopoietic tissues (human immune system Northern blot II) or other tissues (human multiple tissue Northern blot II) were obtained from Clontech. A WWP1 partial cDNA probe was generated by isolating an *Eco*RI−*Not*I fragment from IMAGE Consortium clone 245473. The probe was labeled by random priming with [^{32}P]dCTP, and the blots were hybridized with 2×10^7 cpm of probe in 10 mL of ExpressHyb (Clontech). After stringent washing for 5 min with 2× SSC/1% SDS, followed by 2 h at 68 °C with 0.2× SSC/0.1% SDS, bands were visualized by exposing the blot to a PhosphorImager for 24 h. The blots were stripped and reprobed with a human β-actin probe.

CB3 Cell Transfections and Analysis. CB3 cells are NF-E2 null, murine erythroleukemia cells that fail to express the endogenous β-globin gene following induction of erythroid differentiation (*13*). However, β-globin expression can be rescued by forced expression of p45. The M1 deletion mutant of p45, which fails to rescue globin gene expression, has been described previously (*17*). Three new mutants of the M1 region were constructed by subcloning oligonucleotides into unique *Pst*I and *Sst*I sites of full-length p45. Mutants PPXY-1 and PPXY-2 were generated by substituting alanine residues for amino acids 63−65 (PTY) and 81−83 (PSY) of p45, respectively. The PPXY-1/2 mutant was mutated in both PPXY motifs. Mutants of p45 were subcloned into the expression vector pEF1α-neo (gift of S. Orkin, Harvard Medical School, Cambridge, MA) and transfected into CB3 cells as described previously (*17*). Following selection, individual clones were pooled (five or six clones per pool) and expanded for further analysis. Erythroid differentiation was induced by supplementing the media with 1.8% dimethyl sulfoxide for 72 h. Ribonuclease protection assays for β-globin and β-actin expression and Western blot analysis of p45 protein levels were performed as described previously (*17*). β-Globin expression was quantitated on a PhosphorImager and normalized to β-actin. The mean and standard deviation were determined for six pools per construct. Mutants of NF-E2 were compared to the wild type using the Student's *t* test.

RESULTS

Differential Binding of p45 to WW Domains. The hematopoietic subunit of murine NF-E2, p45, contains two sequences (PPPTY and PPPSY) that would be predicted to interact with WW domains (Figure 1). Indeed, Gavva et al. (*21*) showed that GST−p45 can bind to WW domains from the WW domain-containing proteins NEDD4 and YAP65, in a Far Western assay. Mutagenesis of the PPPPY sequence at amino acids 79−83 of human p45 abolished binding. On the basis of our interest in identifying downstream effectors that mediate transactivation by NF-E2, we developed an ELISA to study the interaction of p45 with WW domains.

We initially overexpressed and purified polyhistidine-tagged murine p45 and measured its ability to interact with WW domains. The WW domains were selected from three representative WW domain-containing proteins, NEDD4, YAP65, and WWP1 (*26*), to determine whether specific WW domains interact preferentially with p45. GST alone, GST fused to an SH3 domain, or GST fused to various WW domains was immobilized in wells of a microtiter plate. The immobilized proteins were incubated with p45 and then incubated with either anti-p45 antibody or anti-GST antibody, followed by incubation with goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase. p45 interacted very weakly with wells lacking the immobilized protein (Figure 2A,B). Similarly, a background signal was seen when p45 was incubated with wells containing immobilized GST or GST−SrcSH3 proteins. SH3 domains bind proline-rich peptides containing the PXXP sequence (*28*). Although this sequence is related to the WW domain binding motif PPXY, it is not a WW domain ligand.

In contrast to those of GST and GST−SH3, variable levels of p45 binding to the GST−WW domain fusion proteins were measured. To ensure that the range of signals in the ELISA was not caused by differences in the amount or accessibility of immobilized proteins, we measured immunoreactivity with an anti-GST antibody. This antibody reacted to a similar degree with all immobilized GST fusion proteins, showing that variable p45 reactivity was not caused by uneven protein immobilization. As the p45 antibody is polyclonal, it is unlikely that variable signals result from differences in the accessibility of the antibody to bound p45. An additional control for the specificity of the immunore-

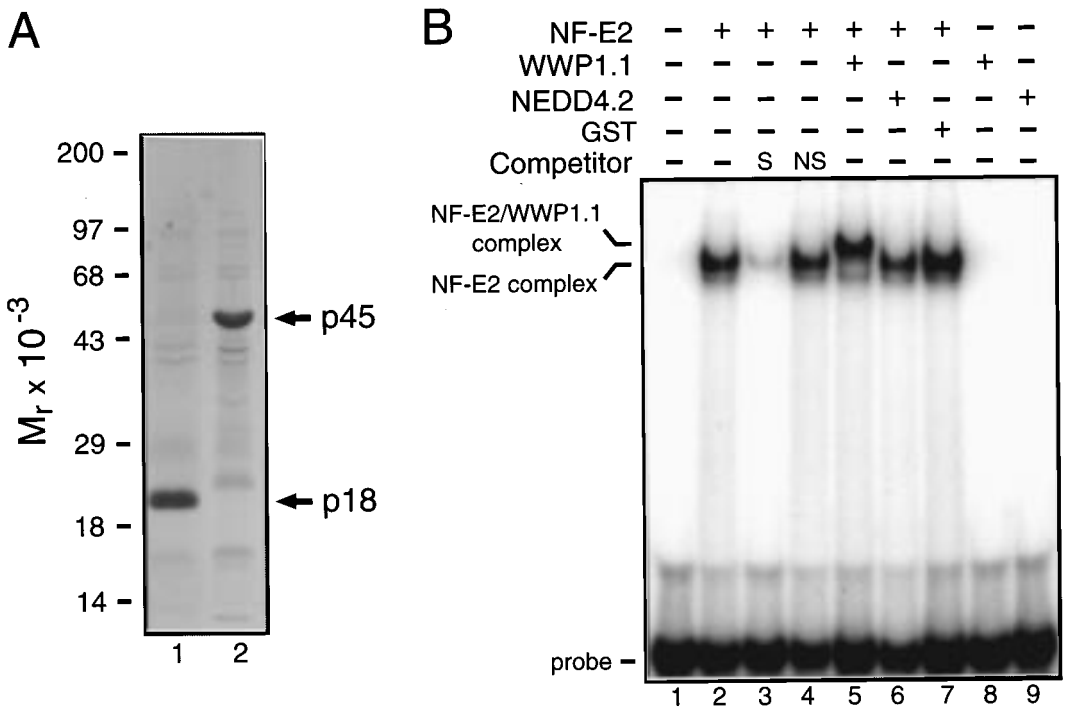

FIGURE 4: Formation of a multimeric complex between DNA-bound NF-E2 and WWP1.1. (A) SDS−PAGE analysis of purified p45 and p18 used to reconstitute NF-E2. Purified polyhistidine-tagged proteins were resolved on an 11% SDS−polyacrylamide gel and stained with Coomassie blue. The positions of molecular weight markers are indicated on the left: lane 1, p18; and lane 2, p45. (B) EMSA analysis. NF-E2 DNA binding was assayed using an oligonucleotide duplex containing a single NF-E2 recognition site, in the presence or absence of a 100-fold stoichiometric excess of cold NF-E2 (S) or an unrelated oligonucleotide with a single upstream stimulatory factor binding site (NS). Certain reaction mixtures contained GST−WWP1.1 or GST−NEDD4.2. Protein−DNA complexes were resolved by EMSA. The positions of unbound probe, the NF-E2−DNA complex, and the NF-E2−WWP1.1−DNA complex are indicated on the left.

action involved incubating immobilized WWP1.1 with an unrelated nuclear protein, type II phosphatidylinositol phosphate kinase (*29*), which also had an amino-terminal polyhistidine tag. After incubation with anti-p45 antibody [Figure 2, WWP1.1 (p)], only a background signal was detected.

Despite the arguments outlined above for the validation of the ELISA, we wanted to use an alternative assay to confirm the relative affinity of p45 for the WW domains. The binding of p45 to the strongest (WWP1.1) and weakest interactors (NEDD4.2) was assessed by Far Western analysis (Figure 2C). While p45 bound strongly to WWP1.1 on the membrane, the interaction with NEDD4.2 was very weak. The signal obtained from p45 binding to NEDD4.2 was only slightly greater than the background resulting from nonspecific binding of [^{125}I]protein A to NEDD4.2. Thus, the relative amount of p45 binding to WWP1.1 and NEDD4.2 was similar to that measured by ELISA.

In contrast to our results, Gavva et al. (*21*) measured strong binding of GST−human p45 to NEDD4.2 by Far Western assay. As only weak binding to NEDD4.2 was observed in our ELISAs, Far Western assays, and EMSAs (Figure 4), the difference between the results may be related to the specific constructs used, our use of murine versus human p45, or our use of lower concentrations of p45 and WW domain proteins in the assays.

The strongest interaction of p45 with WW domains in both assays was detected with WWP1.1. WWP1.1 is one of four WW domains (WWP1.1−1.4) present in the WWP1 protein, a ubiquitin ligase isolated from a human bone marrow cDNA library (*26*). p45 also bound with variable affinities to the three other WW domains of WWP1 (WWP1.3 > WWP1.2 > WWP1.4). WWP1 contains a HECT domain, which represents a ubiquitin ligase catalytic domain (*30*). Moderate to weak binding of p45 was observed with YAP1, NEDD4.1, NEDD4.2, and NEDD4.3 (YAP1 > NEDD4.3 > NEDD4.1 > NEDD4.2). NEDD4.1, NEDD4.2, and NEDD4.3 represent the three WW domains present in the mouse NEDD4 protein, which also is a ubiquitin ligase (*23*). YAP1 is the single WW domain present in the human YAP65 protein (*31*).

High-Affinity Binding of p45 to a WW Domain from the Ubiquitin Ligase WWP1. Certain protein−protein interactions measured in vitro would not be likely to occur in cells due to low-affinity binding. As p45 is a low-abundance transcription factor (*32*), a physiological interaction between p45 and WW domain proteins may require high-affinity binding. Of course, cellular mechanisms involving chaperonins or posttranslational modifications such as phosphorylation could confer a high-affinity protein−protein interaction. We have used a quantitative ELISA to measure the affinity of p45 for WWP1.1. Increasing amounts of p45 were incubated with a constant amount of immobilized WWP1.1. A saturable binding level was achieved with the highest concentrations of p45, which represented a stoichiometric excess of p45 over immobilized WWP1.1 (Figure 3). The binding data were analyzed by nonlinear regression analysis to estimate the K_D for the interaction, assuming that one molecule of p45 interacts with one molecule of the immobilized WW domain. The estimated K_D of 5.7 nM clearly represented a high-affinity interaction.

The results of Figure 3 indicated that most of the immobilized WWP1.1 protein was bound by saturating

concentrations of p45. As saturation was reached at a p45 concentration of 57.6 nM, and 75.8 nM WWP1.1 was present in the well, p45 interacted with at least 76% of the immobilized protein. This value might even be higher, as it is possible that not all WWP1.1 added to the well bound to the plastic, even though the maximal binding capacity of the plastic (0.5 μg of mouse IgG) was not exceeded. This calculation strongly argues against the possibility that only a small amount of the immobilized protein is accessible to p45.

Formation of a Multimeric Complex between WWP1.1, NF-E2, and DNA. Since NF-E2 normally functions as a heterodimeric transactivator complexed to p18, it was important to ask whether functional heterodimers interact with WW domains. Dimerization of b-zip proteins is mediated by the carboxy-terminal leucine zipper and does not require the amino-terminal domains of the heterodimeric partners (*33*). Thus, it seems reasonable to assume that the PPXY motifs of the amino terminus of p45 would be accessible in the NF-E2 heterodimer.

To determine whether DNA-bound NF-E2 can interact with WWP1.1, we reconstituted NF-E2 heterodimers using purified recombinant p45 and p18 (Figure 4A). The functionality of the heterodimers was assessed by EMSA. p45−p18 heterodimers and p18 homodimers bind DNA, whereas p45 homodimers do not. The reconstituted material formed a single protein−DNA complex on an oligonucleotide containing one NF-E2 site (Figure 4B, lane 2). The mobility of the complex resembled the mobility of the complex formed with endogenous MEL cell NF-E2 (data not shown). This observation is consistent with the presence of functional heterodimers in the reconstitute, rather than p18 homodimers, which would have a faster mobility in the gel. Inclusion of GST−WWP1.1 in the binding reaction mixture resulted in a quantitative reduction in the mobility of the NF-E2−DNA complex (Figure 4B, lane 5). In contrast, GST−NEDD4.2 had no effect on the NF-E2 complex (Figure 4B, lane 6), showing that WWP1.1 interacts specifically with DNA-bound NF-E2. A preferential interaction of p45 with WWP1.1 versus NEDD4.2 was also observed in the Far Western assay and ELISA of Figure 2. Importantly, the specificity was identical in solid-state and solution assays. Neither WWP1.1 nor NEDD4.2 bound to DNA in the absence of NF-E2 (Figure 4B, lanes 8 and 9). We were unable to test whether full-length WWP1 interacts with NF-E2, as the intact protein has not yet been overexpressed. The formation of a multimeric complex between DNA-bound NF-E2 and WWP1.1 is consistent with a role for WWP1 or a related WW domain protein as a modulator of NF-E2 function in the nucleus.

Sequence Requirements for High-Affinity Binding of p45 to WWP1.1. The presence of PPXY motifs within the amino-terminal activation domain of p45 suggested that these sequences would be responsible for the high-affinity binding of p45 to WWP1.1. In this regard, Gavva et al. (*21*) mutated PPXY-2 in a GST−p45 fusion protein, and this mutant was incapable of interacting with WW domains in a Far Western assay. We initially tested the specificity of the p45−WW domain interaction using peptides in competitive binding assays. An advantage of using peptides versus protein mutagenesis to delineate sequence determinants of a protein−protein interaction is that mutagenesis can alter protein conformation and thus indirectly affect an interaction.

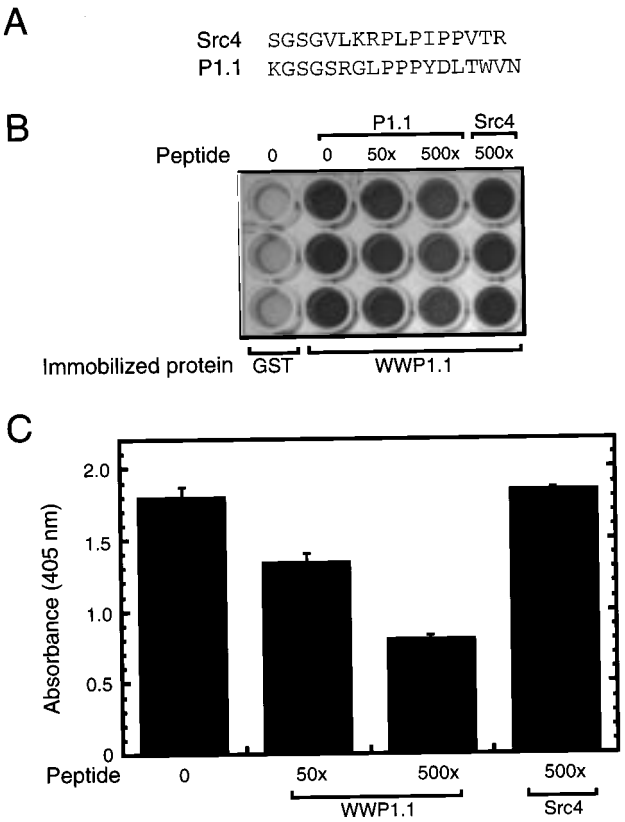

FIGURE 5: Inhibition of p45 binding to WWP1.1 by a WWP1.1-interacting peptide. (A) The peptide sequences are as follows: P1.1, WWP1.1-binding peptide; and Src4, Src SH3 domain binding peptide. (B) The level of binding of p45 to immobilized GST and GST−WWP1 was measured by ELISA, with or without a preincubation of the immobilized proteins with peptides. Peptides were used at a 50- and 500-fold stoichiometric excess (5.5 and 55 μM, respectively) relative to p45. (C) The A_{405} values (mean ± SE, $n = 3$) are plotted for the different conditions. The value for p45 interaction with GST alone (0.27) has been subtracted from other values to yield values representing specific binding.

To determine if WWP1.1 binds p45 at the same site as PPXY peptide ligands, we tested whether a PPXY motif-containing peptide that interacts with WWP1.1 can block the p45−WWP1.1 interaction. This peptide sequence (P1.1) was isolated previously by phage display, on the basis of its interaction with WWP1.1.[2] As a control, we used a proline-rich peptide (Src4) that binds to the Src SH3 domain. A 50- and 500-fold stoichiometric excess (5.5 and 55 μM, respectively) of P1.1 relative to p45 reduced binding by 25.1 and 55.4%, respectively (Figure 5). In contrast, a 500-fold excess of Src4 (55 μM) did not affect binding. Thus, the binding of p45 to the immobilized WW domain occurred at or near the peptide ligand binding site.

Competitive binding assays were also performed to assess the region(s) of p45 required for the WW domain interaction. Two peptides were synthesized spanning either of the two PPXY motifs of p45 (PPXY-1, amino acids 56−70 of murine p45; and PPXY-2, amino acids 74−88) (Figure 6A). A peptide spanning amino acids 21−35 (Cont-1), which did not contain PPXY motifs, was used as a control. A 50- and 500-fold stoichiometric excess (5.5 and 55 μM, respectively) of PPXY-1 reduced the level of binding of p45 to WWP1.1 by 12.0 and 56.2% (Figure 6B,C). Mutation of the PTY residues of the PPXY-1 peptide to AAA (peptide PPXY-

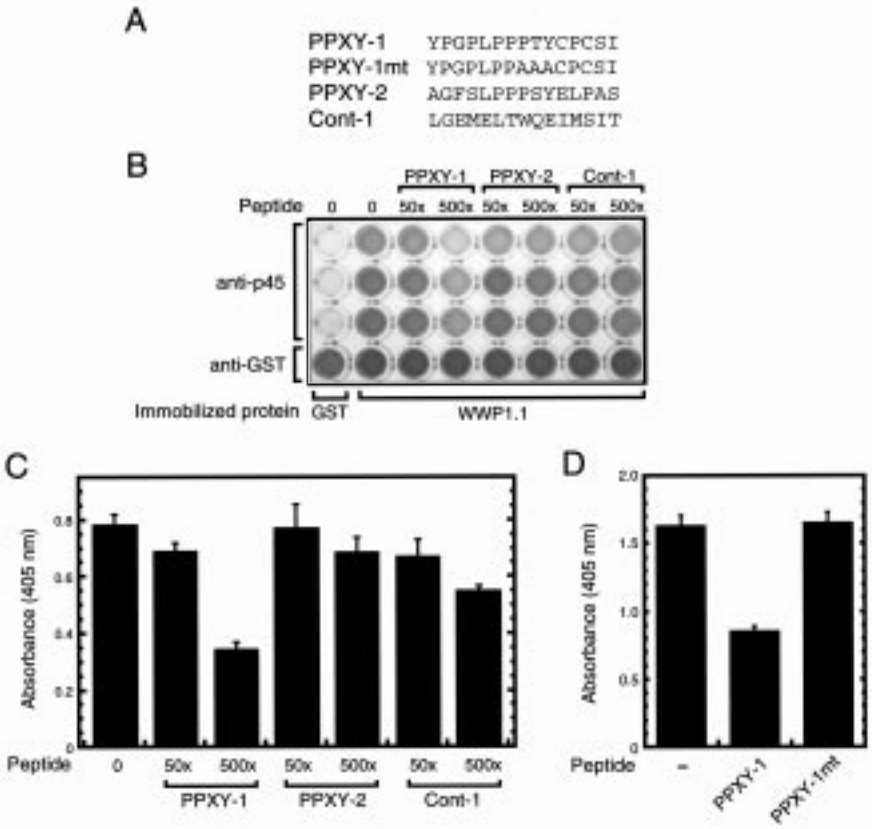

FIGURE 6: Importance of PPXY motifs within the activation domain of p45 for WWP1.1 binding. (A) The peptide sequences are as follows: PPXY-1, amino acids 56−70 of murine p45; PPXY-2, amino acids 74−88 of murine p45; and Cont-1, amino acids 21−35 of murine p45. (B) The level of binding of p45 to immobilized GST and GST−WWP1.1 was measured by ELISA, with or without a preincubation of immobilized proteins with peptides. Peptides was used at a 50- and 500-fold stoichiometric excess (5.5 and 55 μM, respectively) relative to p45. (C) The A_{405} values (mean ± SE, $n = 6$) are plotted for the different conditions. The value for p45 interaction with GST alone (0.15) has been subtracted from other values to yield values representing specific binding. (D) Importance of PTY residues of the PPXY-1 peptide for competition. A peptide was synthesized in which the PXY residues of PPXY-1 were mutated to AAA (PPXY-1mt). The level of binding of p45 to immobilized GST and GST−WWP1.1 was measured by ELISA, with or without a preincubation of immobilized proteins with peptides. Peptides were used at a 500-fold stoichiometric excess (55 μM) relative to p45. The A_{405} values (mean ± SE, $n = 3$) are plotted for the different conditions. The value for p45 interaction with GST alone (0.25) has been subtracted from other values to yield values representing specific binding.

1mt) abolished the ability of PPXY-1 to compete for binding (Figure 6D). A 50- and 500-fold excess (5.5 and 55 μM, respectively) of PPXY-2 only weakly reduced the level of binding (1.7 and 12.7%, respectively). A 50- and 500-fold excess (5.5 and 55 μM, respectively) of Cont-1 had a mild inhibitory effect (14.6 and 29.8%, respectively). The peptides did not affect the reactivity of the anti-GST antibody with WWP1.1, showing that the peptides did not displace WWP1.1 from the microtiter dish. Furthermore, the peptides did not influence the reactivity of p45 with its heterodimeric partner p18 (data not shown). Since the interaction of p45 with p18 is mediated by the carboxy-terminal leucine zippers of p45 and p18, the peptides would not be expected to influence p18 binding. Even though the active peptides (P1.1 and PPXY-1) did not completely inhibit binding at the maximal concentration tested (55 μM), the inhibition was reproducible and quite specific; a K_i for specific binding of a peptide to a protein of ~50 μM is not atypical (*26*). Incomplete competition may be related to the very high affinity of the p45−WWP1.1 interaction (5.7 nM).

Expression of WWP1 in Human Tissues and the Immune System. Although the WWP1 cDNA was isolated from a human bone marrow library (*26*), the expression pattern of WWP1 has not been measured. The murine NEDD4 protein, which also contains WW domains and a HECT domain, has a ubiquitous distribution, being expressed in most embryonic and adult tissues (*34*). A role for WWP1 or a related WW domain protein in p45 function would require the presence of the protein in cells containing p45, such as erythroid and megakaryocytic cells. Thus, we measured the expression pattern of WWP1 by Northern blot analysis, using blots containing mRNA from human hematopoietic tissues and a variety of other tissues. Under high-stringency conditions, the WWP1 cDNA probe only detected a doublet band of ~4.4 kb at variable levels in all tissues (Figure 7A). The blot was reprobed with a human β-actin probe (Figure 7B), which detected the 2.0 kb β-actin transcript in all tissues and the 1.8 kb muscle-specific α-actin transcript in skeletal muscle and heart. The apparent ubiquitous distribution of WWP1 suggests that WWP1 functions in multiple cell types. Importantly, WWP1 was expressed in fetal liver, the major site of fetal erythropoiesis.

Functional Importance of PPXY-1. The high-affinity interaction of WWP1.1 with the activation domain of p45 and the ability of WWP1.1 to interact with DNA-bound NF-E2 suggest that WWP1 or a related protein mediates transactivation by NF-E2. To assess the functional consequences of the NF-E2−WW domain interaction, we generated p45 mutants in which PPXY-1 (PTY) and PPXY-2 (PSY) sequences were mutagenized to AAA, either sepa-

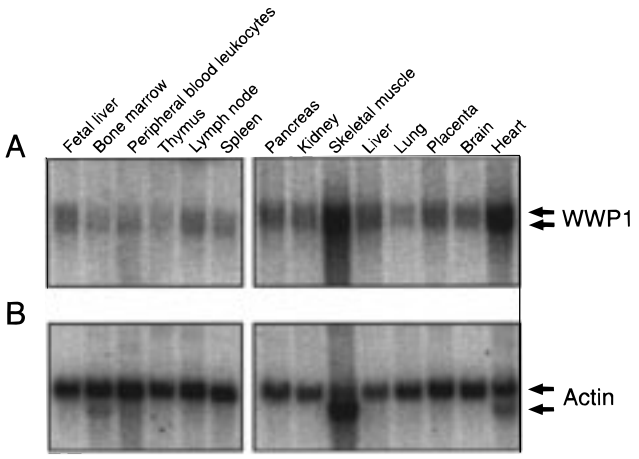

FIGURE 7: Northern blot analysis of WWP1 expression in human tissues. A WWP1 cDNA probe was used to detect WWP1 mRNA transcripts by Northern blotting using blots containing mRNA from human immune tissues or various other tissues. A doublet band at 4.4 kb was detected under high-stringency conditions at variable levels in all tissues (A). The blot was stripped and hybridized with a β-actin probe as a control (B). The 2.0 kb β-actin transcript was detected in all tissues, whereas the 1.8 kb muscle-specific α-actin transcript was detected in skeletal muscle and heart. The size of the transcripts was determined on the basis of the mobility of RNA size standards.

rately or together. Expression constructs containing wild-type and mutant p45 cDNAs were stably transfected into CB3 cells, and pools of five or six clones were studied to determine if the mutations influenced the ability of p45 to rescue endogenous β-globin gene expression.

The M1 mutation, a 42-amino acid deletion within the activation domain of p45, strongly inhibited β-globin gene rescue (96% inhibition) as described previously (Figure 8) (*17*). Mutation of PPXY-1 alone or PPXY-1 and PPXY-2 together strongly inhibited globin gene rescue (73 and 66% inhibition, respectively). In contrast, mutation of PPXY-2 alone only had a small effect, which was not statistically significant (19% inhibition). The inhibitory effect of the PPXY-1 mutation suggests that the absence of the PTY sequence in the M1 mutant is partially responsible for the low activity of M1. Western blot analysis of wild-type and mutant p45 protein levels showed that the proteins were expressed in similar amounts in the different pools (data not shown). Taken together with the results of Figure 6 showing that the PPXY-1, but not the PPXY-2, peptide inhibits the binding of p45 to WWP1.1, PPXY-1 is important for both efficient transactivation and WW domain binding. These results are consistent with the hypothesis that PPXY-1 interacts with a WW domain protein, which potentiates transactivation by NF-E2.

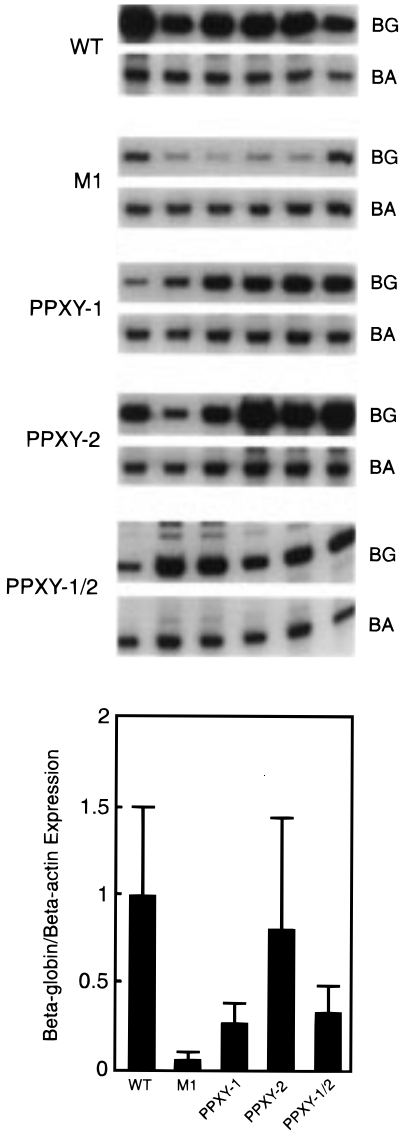

FIGURE 8: The PPXY-1 motif is required for full transcriptional activation by NF-E2. Endogenous β-globin (BG) and β-actin (BA) expression was measured by ribonuclease protection assay in pools of CB3 cells stably expressing wild-type (WT) NF-E2 or mutants of NF-E2 (six pools for each construct). The M1 deletion mutant is shown in Figure 1. Mutants PPXY-1 and PPXY-2 were generated by substituting alanine residues for amino acids 63−65 (PTY) and 81−83 (PSY) of NF-E2, respectively. Mutant PPXY-1/2 was mutated in both PPXY motifs. The levels of β-globin and β-actin mRNA expression were quantitated with a PhosphorImager and expressed as relative units of β-globin/β-actin (mean ± standard deviation, $n = 6$): M1 mutant, $p = 0.006$; PPXY-1 mutant, $p = 0.015$; PPXY-2 mutant, $p = 0.575$; and PPXY-1/2 mutant, $p = 0.021$.

DISCUSSION

We have shown that the PPXY-1 motif of p45, located within the activation domain, is important for WW domain binding and transactivation. PPXY-1 mediated binding to several WW domains, with the highest-affinity binding being to WWP1.1 of the WWP1 ubiquitin ligase. Strong WWP1.1 binding was measured in three independent assays: ELISA, Far Western, and EMSA. In a related study, we have shown that PPXY-1 is not required for CBP/p300 binding to p45,[3] and thus, disruption of CBP/p300 binding cannot explain inhibition by the PTY to AAA mutation.

A key question arising from our work is what is the endogenous protein that recognizes PTY and mediates increased transactivation. Several candidates include the WW domain proteins, NEDD4 (*34*), RPF1 (*35*), and WWP1 (*26*), which are ubiquitin ligases, and the WW domain protein, Pin1, a nuclear peptidyl−prolyl isomerase that regulates mitosis (*36*). An intriguing possibility is that NF-E2 recruits a ubiquitin ligase to chromatin, analogous to HAT recruitment by transcription factors (*37*).

Ubiquitin ligases are essential components of the cellular ubiquitinating machinery. The ligase, also called an E3 enzyme, functions coordinately with E1 (activating) and E2

(conjugating) enzymes to conjugate a single residue or multiple residues of ubiquitin to protein substrates (reviewed in ref *38*). Polyubiquitination provides a signal for a protein to be degraded by the proteasome and is an important regulator of protein stability. Proteins can also be monoubiquitinated, and this appears to have regulatory functions distinct from controlling protein stability. Monoubiquitination has been reported to be necessary for the assembly of a ribosomal protein into the ribosome (*39*). This activity may reflect a chaperonin function of ubiquitin that facilitates the assembly or disassembly of macromolecular structures. The localization of ubiquitin ligases to the cytoplasm and nucleus suggests that they function in multiple cellular compartments (*40*).

Two lines of evidence support a role for ubiquitination in transactivation. First, histones H2A and H2B can exist as monoubiquitinated species in chromatin (*41*, *42*), and there is a correlation between the presence of these histone variants and transcriptionally active chromatin (*43*). Second, a human ubiquitin ligase, RPF1, potentiates transactivation by the progesterone and glucocorticoid receptors (*35*), which are ligand-activated transcription factors that induce chromatin remodeling (reviewed in ref *44*). However, mutation of the cysteine residue that forms a thioester with ubiquitin did not abolish potentiation, suggesting that enzymatic activity was not required.

On the basis of the binding and functional studies described herein, we hypothesize that a WW domain protein potentiates transactivation by NF-E2. The ubiquitous distribution of WWP1 is not inconsistent with such a function, as there are other examples of tissue-specific transcription factors that utilize ubiquitous coactivators (*45*). Is the WW domain protein interaction a mechanism used commonly by other hematopoietic transcription factors? We have found PPXY motifs within multiple transcription factors, including the LDB1 protein, which are conserved between *Xenopus* and mice. LDB1 is a LIM domain binding protein that interacts with TAL1 (*46*), a basic helix−loop−helix transcription factor (*47*, *48*), which controls hematopoiesis (*49*, *50*). The interaction of LIM domain proteins with DNA-bound TAL1/E2A heterodimers may influence TAL1 DNA binding specificity (*51*). More recently, Visvader et al. (*46*) showed that LDB1 opposes the hematopoietic stimulatory activity of TAL1. Like p45, one can hypothesize that DNA-bound TAL1 heterodimers interact with LDB1, which then recruits a WW domain protein. It will be important to identify nuclear WW domain proteins in megakaryocytes and erythroid cells and to determine the specific protein(s) that functionally interacts with NF-E2.

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