Interaction of WW Domains with Hematopoietic Transcription Factor p45/NF-E2 and RNA Polymerase II*  

(Received for publication, July 8, 1997)  
Narendra R. Gavva, Rama Gavva, Kira Ermekova, Marius Sudol, and C.-K. James Shen††  
From the †Section of Molecular and Cellular Biology, University of California, Davis, California 95616, the §Department of Biochemistry, Mount Sinai School of Medicine, New York, New York 10029, and the ¶Institute of Molecular Biology, Academia Sinica, Taipei 11529, Republic of China  

NF-E2 is an erythroid-specific transcription factor required for expression of several erythroid-specific genes. By Far-Western blotting and yeast two-hybrid assay, we demonstrate that p45, the large subunit of NF-E2, is capable of binding to a specific set of WW domain-containing proteins, including the ubiquitin ligase hRPF1. This binding is mediated through the interaction between the WW domains and a PDY motif located within the amino-terminal region of p45. Interestingly, the carboxyl-terminal domain of mammalian RNA polymerase II binds a similar set of WW domains to which p45 interacts with. We discuss the data in terms of possible new pathways through which the processes of transcriptional regulation by NF-E2 could be regulated in erythroid and megakaryocyte cells.

NF-E2 is an obligate heterodimer of basic leucine zipper polypeptides consisting of a larger p45 polypeptide and a smaller subunit belonging to the p18/Maf family (1–5). Of the two subunits of NF-E2, expression of p18/Maf is ubiquitous, while that of p45 is restricted to the erythroid and megakaryocyte cell lineages (1, 6). Indeed, intact p45 gene and its expression are required for transcriptional regulation of globin genes (Refs. 1 and 7–9 and references therein) as well as for normal differentiation of the megakaryocytes (10).

As demonstrated below, p45 indeed could interact with several of these proteins, in vivo and in vitro, through specific interaction between the PDY motif at amino acids 78–83 and the different WW domains. We also show that RNA pol II-CTD and p45 bind similar WW domains, including the mNEDD4-WW2. The study has suggested new pathways through which the processes of transcriptional activation by the NF-E2 molecule could be regulated in erythroid and megakaryocyte cells.

EXPERIMENTAL PROCEDURES

Bacterial Expression Plasmids—p45 cDNA was amplified from K562 RNA by RT-PCR (27) with primers A and B and blunt-ended cloned into the EcoRI site of pGEX-2T. To create two fragments from the p45 molecule, two fragments were first generated by PCR amplification of p45 cDNA with primer pairs B/C and A/D, respectively. These two fragments were then used as the templates for PCR with primers A and B. The final product, p45(P81A,Y83G), was blunt-ended cloned into the Ava I site of pGEX-2T. The p45 protein expressed from this plasmid contains two amino acid substitutions in the PDY motif: Pro→Ala at 81 and Tyr→Gly at 83. Cloning of GST-WBP1-PY was described previously (18), hYAP-WW, mYAP-WW1, mYAP-WW2, mNEDD4-WW1, mNEDD4-WW2, mNEDD4-WW3, yEss1-WW, and hDys-WW were individually PCR-amplified from the appropriate cDNAs with primer pairs E/F, G/H, I/J, K/L, M/N, O/P, Q/R, and S/T, respectively, and ligated at the BamHI-EcoRI sites of pGEX-2TK. Protein kinase A-phosphorylatable GST-CTD fusion protein was kindly provided by Michael E. Dahmus (University of California, Davis, CA).

Sequences of all the primers (A–Z) used in this study are available upon request. Authenticity of the clones was confirmed by restriction enzyme digestion and DNA sequencing. p45 and p45(P81A,Y83G) were

mones. NF-E2 binding to its cognate sequence also induces local nucleosome disruption (15, 16). The NH2-terminal region of p45 has been implicated as the transcriptional activation domain of NF-E2 (9), and it contains 18% of proline residues. Interestingly, during data base search and visual comparison of the p45 sequence, we have noticed the sequence PPPPP located at amino acids 79–83 of human p45. In mouse, it is PPSYS. This sequence fits the consensus of the so-called “PDY” motifs, XPPXY, in which P is a proline, Y is a tyrosine, and Xs are any amino acid (17, 18; Fig. 1). The PY motifs are ligands capable of binding to the WW domains, which in turn were first identified in the proto-oncogene Yes-associated protein (YAP),1 dystrophin, transcriptional regulator FE65, and others (19). These domains are of the length 38 amino acids, and they contain β strands grouped around four aromatic positions (17, 20). Two of these positions are most frequently occupied by tryptophans, hence the name “WW” domain was given. The WW domains are found in a number of unrelated proteins, including human and mouse YAP (hYAP, mYAP), human dystrophin (hDys), human ORF1, yeast Rsp5, yeast Ess1 (yEss1), fission yeast Pub1, mouse NEDD4 (mNEDD4), human RPF1/NEDD4 (hRPF1), and FE65 (reviewed in Ref. 17). The functions of these proteins range from cell cycle control of yEss1 (21), cell cycle control and ubiquitin ligase activity of Pub1 (22), to the ubiquitin ligase activity of Rsp5 (23, 24) and mNEDD4 (25), and transcriptional co-activator properties of the hRPF1 (26).

As demonstrated below, p45 indeed could interact with several of these proteins, in vivo and in vitro, through specific interaction between the PDY motif at amino acids 78–83 and the different WW domains. We also show that RNA pol II-CTD and p45 bind similar WW domains, including the mNEDD4-WW2. The study has suggested new pathways through which the processes of transcriptional activation by the NF-E2 molecule could be regulated in erythroid and megakaryocyte cells.

* This work was supported by the United States Public Health Service, National Institutes of Health Grants DK29800 (to C.-K. J. S) and CA45757 and CA01605 (to M. S.), the National Science Council, and the National Health Research Institute, Taipei, Republic of China. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 916-752-8860; Fax: 916-752-3085; E-mail: ckshen@ccvax.sinica.edu.tw.

1 The abbreviations used are: YAP, Yes kinase-associated protein; NEDD4, NPC-expressed, developmentally down-regulated 4; hRPF1, human receptor potentialization factor 1; GST, glutathione S-transferase; WBP1, WW domain-binding protein 1; GBD, Gal4 DNA-binding domain; GAD, Gal4 activation domain; NIPPI, NF-E2-interacting polypeptide 1; CTD, COOH-terminal domain; pol, polymerase; RT-PCR, reverse transcription-polymerase chain reaction.
p45 Specifically Interacts with a Subset of WW Domains in Vitro—The interaction between p45 and different WW domains is analyzed by Far-Western blot analysis. As a positive control, we first studied the binding of the PY motif of WBP-1 to the WW domains by this approach (Fig. 2). The WBP-1 PY motif, of the WW domains by this approach (Fig. 2). The WBP-1 PY motif, of the WW domains by this approach (Fig. 2). The WBP-1 PY motif, of the WW domains by this approach (Fig. 2). The WBP-1 PY motif, of the WW domains by this approach (Fig. 2). The WBP-1 PY motif, of the WW domains by this approach (Fig. 2). The WBP-1 PY motif, of the WW domains by this approach (Fig. 2). The WBP-1 PY motif, of the WW domains by this approach (Fig. 2). The WBP-1 PY motif, of the WW domains by this approach (Fig. 2). The WBP-1 PY motif, of the WW domains by this approach (Fig. 2). The WBP-1 PY motif, of the WW domains by this approach (Fig. 2).

Far-Western analysis demonstrates that p45 indeed binds to a subset of the WW domains tested (Fig. 3). As shown in Fig. 3B, binding between p45 and mNEDD4-WW2 is the strongest (lane 5, Fig. 3B), p45 also binds efficiently to hYAP-WW2 and hYAP-WW1 (lanes 1 and 2, Fig. 3B), but less efficiently to mYAP-WW2 (lane 2, Fig. 3B). However, p45 does not bind to the other WW domains tested (lanes 4, 6, and 7, Fig. 3B) as well as GST-WBP1-PY or GST (lanes 8 and 9, Fig. 3B).

Interactions between p45 and WW Domains Is Modulated through the PY Motif of p45—To test whether PY motif at amino acids 79–83 of p45 is essential for the observed interaction between p45 and the WW domains, we then carried out Far-Western blot analysis using the GST-p45 and GST-p45(P81A,Y83G) mutant as the hybridization probes. As shown in Fig. 3C, the two amino acid substitutions at positions Pro81 and Tyr83 of p45 abolish its interaction with all of the tested WW domains (Fig. 3C). Far-Western blot hybridization with cold p45 gave the same results.2 Note that in the blot of Fig. 3C, a NIPP1-(1–203) polyepptide fused to GST was included as a positive control. NIPP1 is a novel p45-binding protein,3 and its interaction with p45 is not modulated through the PY motif of p45.

In Vivo Interaction of p45 with WW Domain-containing Proteins—The yeast two-hybrid system was used to test whether p45 could interact in vivo with two of the WW domain-containing proteins, hRFP1 and hYAP. hRFP1 is the human homolog of mNEDD4 (19, 24, 25). Plasmids expressing GBD only, GBD fusion of p45 (38–115), and GBD fusion of p45 (38–115: P81A,Y83G) were transformants into yeast cells, but not with p45-(38–115: P81A,Y83G) mutant as the hybridization probes. As shown in Fig. 3C, the two amino acid substitutions at positions Pro81 and Tyr83 of p45 abolish its interaction with all of the tested WW domains (Fig. 3C). Far-Western blot hybridization with cold p45 gave the same results.2 Note that in the blot of Fig. 3C, a NIPP1-(1–203) polyepptide fused to GST was included as a positive control. NIPP1 is a novel p45-binding protein,3 and its interaction with p45 is not modulated through the PY motif of p45.

2 N. R. Gavva, data not shown.
3 N. R. Gavva and C.-K. J. Shen, unpublished data.
P81A,Y83G) containing the mutated PY motif.

RNA Polymerase II-CTD Binds the Similar Subset of WW Domains as p45/NF-E2—Recently it was shown that yeast RNA pol II is the substrate of the ubiquitin ligase Rsp5. This reaction is mediated through binding between the CTD domain of RNA pol II and the NH2-terminal part of Rsp5 (24). RNA pol II-CTD contains several py motifs with a consensus “XSPXY.” We have tested whether the CTD of mammalian RNA pol II could also bind mNEDD4, the mammalian homolog of Rsp5, through one of the three WW domains of the latter. Indeed, as shown in Fig. 5, CTD of mouse RNA pol II binds efficiently to the YAP WW domains as well as to mNEDD4-WW2, but not to the WW1 and WW3 of mNEDD4, yEss1-1WW, hDys-WW, or GST (Fig. 5B). This clearly indicates that mammalian RNA pol II-CTD could bind to the mammalian ubiquitin ligase. Furthermore, this binding, in mouse or humans, is most likely mediated through the WW2, but not with the other WW domains of the ubiquitin ligase(s) (also see “Discussion”). It is interesting to note that RNA pol II-CTD binds the same set of WW domains as p45, although the affinities toward different WW domains are different between the two probes (compare Figs. 3B and 5B).

DISCUSSION

NF-E2 activates transcription of the globin genes through binding to the locus control regions of the globin loci (reviewed in Refs. 32 and 33). Alternatively, it can activate transcription of genes such as porphobilinogen deaminase through binding to their upstream promoters (11, 34). Similar to the other sequence-specific, DNA-binding transcription factors (reviewed in Refs. 35 and 36), these activation processes by NF-E2 must involve the interaction of NF-E2 with other transcription activators or co-activators, as exemplified in Ref. 14. The present identification of the PY motif of p45 as a ligand for specific WW domains has revealed a new class of interacting proteins through which the trans-activation function of NF-E2 could be regulated.

Specific Interaction between p45 and the WW Domains—Far-Western blot analysis demonstrates that the p45 subunit of NF-E2, as well as the CTD of RNA pol II, binds specific sets of the WW domains (Figs. 3 and 5). The various WW domains of several WW domain-containing proteins and the relative strength of their interaction with p45 and RNA pol II-CTD are listed in Table I.

Sequence specificities affecting the interaction between different PY motifs and WW domains have been studied by biochemical and structural approaches (18, 20, 37, 38). In particular, NMR analysis of the hYAP-WW domain-bound WBP1-PY motif indicated that Trp39 in the WW domain contacts the 2nd and 3rd prolines of PPPYY. In addition, Leu30 and His32 contact the tyrosine (20). Binding site for the peptides is a large hydrophobic patch on the WW domain surface formed by side chains of Tyr28, Leu30, Trp39 and by the methyl groups of one or more threonines at positions 36–38. Consistent with the above, and the extensive data on site-directed mutagenesis of the PY motif of WBP1 (37), mutation at the 3rd proline and the tyrosine of the p45 PY motif abolished its binding with the WW domains (Fig. 3C).

A close examination of the sequences listed in Table I suggests that the presence of three clustered threonines at 36–38 is one of the major determinants for a strong interaction between a WW domain and p45. The binding affinity is drastically reduced when one of the threonine's is substituted with different amino acids. Furthermore, a change of Leu30 to Val30 in mNEDD4-WW2 appears to result in a relative higher affinity toward p45, in comparison with those of mYAP-WW1 and hYAP-WW (Table I). Following the above reasoning, we anticipate that it is the WW2 domain, but not WW1, WW3, or WW4 of hRPF1, that is responsible for the interaction in vivo between p45-(38–115) and N-hRPF1 (Fig. 4).

Functional Implications of p45-WW Interaction—Although the exact functions of the motif-specific interaction between p45 and WW domains await further investigations, it is possible that the WW domain-containing proteins are involved in the regulation of the NF-E2 activity via one or more of the following mechanisms. First, p45 interacts efficiently with YAP (Figs. 3 and 4). Since YAP can associate with the SH3 domain of the Yes tyrosine kinase, it is possible that YAP-like protein(s) may target some of its WW domain-binding proteins, such as p45 and RNA pol II, for phosphorylation by kinases. Thus, activity of NF-E2 could be regulated by phosphorylation at specific residues. Second, hRPF1 has been shown to function as a co-activator/potentiator for hormone receptor-mediated transcriptional activation (26). Thus, hRPF1 or its family members may act, through direct binding to p45, as co-activators or even repressors for NF-E2 regulated transcription in erythroid and/or megakaryocytic cells.

Third, like its homologs, Rsp5 (23, 24) and mNEDD4 (25), hRPF1 may also possess ubiquitin ligase activity. As demon-
stratot (24), Rsp5 and its homologs, when bound to RNA pol II-CTD, may ubiquitinate the RNA polymerase II. Furthermore, p45 interacts with the RNA pol II-CTD, may ubiquitinate the RNA polymerase II. Such a tertiary complex may play a role during the processome degradation pathway. It is also possible that through utilization of different WW domains, Rsp5 homologs such as the mNEDD4/hRPF1 or others could serve as a binding bridge between NF-E2 and RNA polymerase II. Such a tertiary complex may play a role during transcriptional activation of erythroid-specific genes by NF-E2. Alternatively, DNA-bound p45 may recruit mNEDD4/hRPF1-like proteins to ubiquitinate nearby histones, a reaction that may lead to an open chromatin structure (39, 40). p45 and the bound WW domain proteins may also form tertiary complexes with other nuclear proteins such as the protein kinases of splicing factors, as identified recently (41).

Finally, it should be noted that there is an increasing number of WW domain-containing proteins found in cells carrying out diverse regulatory functions (42). Indeed, three more such proteins belonging to the Rsp5/NEDD4 family have recently been cloned by a ligand screening procedure (38), and by the yeast two-hybrid system.

Acknowledgment—We thank Dr. Mike Dahmus for providing the recombinant GST-CTD protein and Xin Chen for helpful discussions.

REFERENCES

1. Andrews, N. C., Erdjument-Bromage, H., Davidson, M. B., Tempst, P., and Orkin, S. H. (1995) Nature 373, 722–728.
2. Nye, P. A., Andrews, N. C., Jane, S. M., Safer, B., Purucker, M. E., Waremowicz, S., Morton, C. C., Goff, S. C., Orkin, S. H., and Nienhuis, A. W. (1993) Mol. Cell. Biol. 13, 568–572.
3. Chien, C. T., Bartel, P. L., Sternglanz, R., and Fields, S. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7454–7458.
4. Grosveld, F., Dillon, N., and Higgs, D. (1993) Bailliere's Clin. Haematol. 6, 31–55.
5. Mignotte, V., Eleouet, J. F., Raich, N., and Romeo, P.-H. (1989) Eur. J. Biochem. 231, 271–281.
6. Grosveld, F., Dillon, N., and Higgs, D. (1993) Bailliere's Clin. Haematol. 6, 31–55.

TABLE I

| Protein/species | Position | Sequence of WW domains | Accession no. | Binding to p45 NF-E2 | Binding to RNA pol II-CTD |
|-----------------|----------|------------------------|---------------|---------------------|--------------------------|
| mYAP-1          | 151      | VPLPGWEMAKTKS-GQRFY1HNDQDTITWQP1PRKAMS | X80508 | +++ | +++ |
| mYAP-2          | 218      | G1FLPGWEM1AQMD-GEVY1YNHRRKS1WDPFLPPRF | X80508 | + | + |
| hYAP            | 171      | VPLPGWEM1AQMD-GEVY1YNHRRKS1WDPFLPPRF | X80508 | + | + |
| mNEDD4-1        | 139      | SGLPGWEGQVDKL-GRTY1YNHRSST1WQDPRPFDODI | U96635 | +++ | +++ |
| mNEDD4-2        | 295      | SGLPGWEGQVDKL-GRTY1YNHRSST1WQDPRPFDODI | U96635 | +++ | +++ |
| mNEDD4-3        | 350      | SGLPGWEGQVDKL-GRTY1YNHRSST1WQDPRPFDODI | U96635 | ND | |
| yEssa1          | 29       | TGLPTFLWY1SSK1REYF1NEE1WQDPRPFDODI | P23286 | | |
| hDys            | 3052     | TSVQPGWERA1SPN-KVY1YNHETS1WDPF1TMLY | P11532 | | |
| hRPF1-1         | 218      | SGLPGWEGQVDKL-GRTY1YNHRSST1WQDPRPFDODI | D42055 | | |
| hRPF1-2         | 375      | SGLPGWEGQVDKL-GRTY1YNHRSST1WQDPRPFDODI | D42055 | | |
| hRPF1-3         | 448      | SGLPGWEGQVDKL-GRTY1YNHRSST1WQDPRPFDODI | D42055 | | |
| hRPF1-4         | 500      | SGLPGWEGQVDKL-GRTY1YNHRSST1WQDPRPFDODI | D42055 | | |
| yRsp5-1         | 228      | GRLPGWEGQVDKL-GRTY1YNHRSST1WQDPRPFDODI | L11119 | ND | |
| yRsp5-2         | 331      | GRLPGWEGQVDKL-GRTY1YNHRSST1WQDPRPFDODI | L11119 | | |
| yRsp5-3         | 387      | GRLPGWEGQVDKL-GRTY1YNHRSST1WQDPRPFDODI | L11119 | | |

ND indicates the amino acid position in WW domain as shown for hYAP-WW in Ref. 20.

**a** Numbers indicate the amino acid position in WW domain as shown for hYAP-WW in Ref. 20.

**b** ND, not determined.

**c** p45 (38–115) interacted with hRPF1 (1–553) in yeast two-hybrid system.

**d** The Nterminal of RSP5 binding with RNA pol II-CTD contains all of the three WW domains listed (see Ref. 24).

---

4 X. Chen and C.-K. J. Shen, unpublished data.