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Functional properties of the N-terminal region of progesterone receptors and their mechanistic relationship to structure☆

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Abstract

Progesterone receptors (PR) are present in two isoforms, PR-A and PR-B. The B-upstream segment (BUS) of PR-B is a 164 amino acid N-terminal extension that is missing in PR-A and is responsible for the functional differences reported between the two isoforms. BUS contains an activation function (AF3) which is defined by a core domain between residues 54–154 whose activity is dependent upon a single Trp residue and two LXXLL motifs. We have also identified sites both within and outside of BUS that repress the strong synergism between AF3 and AF1 in the N-terminal region and AF2 in the hormone binding domain. One of these repressor sites is a consensus binding motif for the small ubiquitin-like modifier protein, SUMO-1 (^{387}IKEE). The DNA binding domain (DBD) structure is also important for function. When BUS is linked to the glucocorticoid receptor DBD, AF3 activity is substantially attenuated, suggesting that binding to a DNA response element results in allosteric communication between the DBD and N-terminal functional regions. Lastly, biochemical and biophysical analyses of highly purified PR-B and PR-A N-terminal regions reveal that they are unstructured unless the DBD is present. Thus, the DBD stabilizes N-terminal structure. We propose a model in which the DBD through DNA binding, and BUS through protein–protein interactions, stabilize active receptor conformers within an ensemble distribution of active and inactive conformational states. This would explain why PR-B are stronger transactivators than PR-A.

Keywords: Progesterone receptor; Transcription; Synergism; Cofactors; Structure; Activation domain; Repression; SUMO

1. Introduction

Steroid receptors regulate a complex network of pathways controlling development, differentiation and homeostasis. These nuclear proteins were defined as having a modular structure with distinct domains that can function independently. Specific functions have been ascribed to each domain, including hormone binding, DNA binding and transcriptional activation. With the exception of estrogen receptor α (ERα), the high degree of sequence similarity between DNA binding (DBD) and hormone binding (HBD) domains within the steroid receptor family belies the diverse array of gene promoters and metabolic pathways that they regulate [1]. We and others have speculated that this diversity is subserved by the N-terminal regions of the receptor proteins, which are highly dissimilar in both sequence (<15% sequence identity) and size (184 and 566 amino acids for ERα and the progesterone receptor B-isoform (PR-B), respectively). Several recent studies have provided suggestive functional and structural evidence that N-terminal regions do indeed play a critical role in determining which gene promoters and, therefore, which functional pathways are regulated by these receptors [2–7].

PR provide an ideal model system to probe the mechanistic details by which receptor N-terminal regions modulate function. First, they exist in two isoforms, PR-A and PR-B, which have large N-terminal regions of 402 and 566 amino acids, respectively, that are identical in sequence except that PR-A are missing the far 164 residue N-terminal B-upstream segment (BUS) region. This conveniently provides a discrete physical entity upon which to focus functional, biochemical and biophysical studies. Transcription activation as well as repression functions have been identified within the N-terminal regions, some of which are unique to the BUS

Abbreviations: BUS, B-upstream segment; AF, activation function; PR, progesterone receptors; ER, estrogen receptors; GR, glucocorticoid receptors; AR, androgen receptors; DBD, DNA binding domain; HBD, hormone binding domain; NT, N-terminal; BDN, BUS-DBD-nuclear localization signal; PRE, progesterone response element; GRE, glucocorticoid response element; ERE, estrogen response element; IF, inhibitory function; SUMO, small ubiquitin-like modifier

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region present in PR-B [8–12]. Despite similarities between the two PR isoforms, the transcriptional phenotype of PR-A and PR-B on both synthetic and endogenous promoters is very different. PR-B are generally strong transcriptional activators, while PR-A are weak activators and exhibit stronger repressor properties [13,14]. Moreover, recent gene array studies in our laboratory show that PR-A and PR-B regulate relatively distinct gene programs in vivo [15,16].

In the present study, we have precisely mapped activator and repressor activities within the N-terminal regions of both receptors, which, we propose, are responsible for their diverse functional responses. This includes delineation of the core activation domain within activation function 3 (AF3) of BUS that is required for the high degree of AF3 synergism with AF1 and AF2 domains [3]. We also identified a consensus binding motif for the small ubiquitin-like modifier protein, SUMO-1, that tightly controls AF synergism in both isoforms [17,18]. Finally, we initiated biochemical and biophysical studies using highly purified N-terminal regions of both PR-A and PR-B to assess their structural properties. Our findings show that the N-terminal regions are largely unstructured. However, these regions assume an ordered conformation when the DNA binding domain is present, and assume further structural changes when the proteins are bound to a DNA response element. Additionally, the presence of BUS reduces heterogeneity in the ensemble distribution of N-terminal conformations, suggesting that there is an enrichment of active conformers when BUS is present. We speculate that these data provide a mechanistic basis to explain functional differences between PR-A and PR-B.

2. Materials and methods

2.1. Recombinant plasmids and site-directed mutagenesis

Human PR-B (pSG5-hPR1), PR-A (pSG5-hPR2) and ERα (pSG5-ERα) were kind gifts of P. Chambon. Construction of NT-B, NT-A, and DBD in the pSG5 mammalian expression vector have been described [9]. The site-directed mutants in PR-B, mL1, mL2, mL1/mL2, and $W^{140}A$ were constructed as described [3]. Construction of site-directed mutants PR-B $K^{388}R$ and PR-A $K^{388}R$ have been described [4]. PR-B K^5W/K^7W were constructed by PCR heteroduplex-based mutagenesis and screened by introducing a translationally silent *Xho*I restriction site adjacent to the mutated residues. $BD_{GR}N$ was constructed by replacing the *Rsr*II/*Kpn*I fragment (residues 556–645) in BDN with the corresponding human GR DBD fragment (residues 430–519). $BD_{GR/PR}N$ was constructed by introducing translationally silent *Bgl*II and *Pst*I sites at the borders of the core PR DBD (residues 565 and 630) and subcloning annealed oligonucleotides representing the corresponding N- and C-terminal GR flanking sequences (residues 430–440 and 507–519, respectively) into the resulting *Rsr*II–*Bgl*II and *Pst*I–*Kpn*I sites. The plasmid pJJ521 [19] was constructed by inserting an *Eco*RI fragment containing the Gal 1.10 promoter from yeast strain (Sc4816) into the plasmid Yeplac181 [20]. The plasmid pJJ/BDN was constructed by inserting BDN downstream of the galactose inducible promoter.

2.2. Random mutagenesis

We used a random mutagenesis technique in which reduced/loss of function mutant AF3 cDNAs were selected. The 492bp cDNA encoding BUS was mutagenized by a modified PCR technique [21] that randomly introduces base substitutions into the amplified cDNA target. Plasmid DNA from random clones was sequenced to determine an average mutation rate of 2.5% and a range of 6–15 nucleotide changes per fragment. Remaining PCR product was ligated into the pJJ(BDN) vector. Aliquots of the ligation mix were cotransformed with the reporter plasmid, pSX26.1, and the control plasmid, pGA702, into the yeast strain RS299 by the lithium acetate method [22]. Yeast colonies were assayed for AF3 activity by the agar overlay assay method [23]. A total of 35,000 yeast colonies were screened and 210 colonies demonstrated the reduced/loss of function phenotype. Mutant BUS plasmids were selected and sequenced. Sequence analysis/alignment and protein translation/alignment against the wildtype BUS region of PR was performed using the DNA* analysis suite of software programs (DNASTAR Inc., Madison, WI).

2.3. Protein expression

Expression of all cDNA constructs was confirmed by transient transfection into COS-1 or HeLa cells as described [3]. Cell extracts were prepared from transfected cells; proteins were resolved by SDS-PAGE; transferred to nitrocellulose, probed with monoclonal or polyclonal antibodies; and visualized by enhanced chemiluminescense as described [3].

2.4. Transcription assays

HeLa cells were transfected by calcium phosphate co-precipitation with designated amounts of expression vectors and a PRE_2-TATA or ERE_2-TATA luciferase promoter–reporter plasmid as described [3,4]. Cells were treated or not with hormone, harvested, lysed, and luciferase activity was measured and normalized to luciferase activity generated from a cotransfected SV40 *Renilla* luciferase plasmid as described [3].

3. Results

3.1. AF3 synergizes with AF1 and AF2 and maps to specific residues within BUS

In Fig. 1, we compared transcriptional activities of PR constructs expressing one or more AFs, using a synthetic

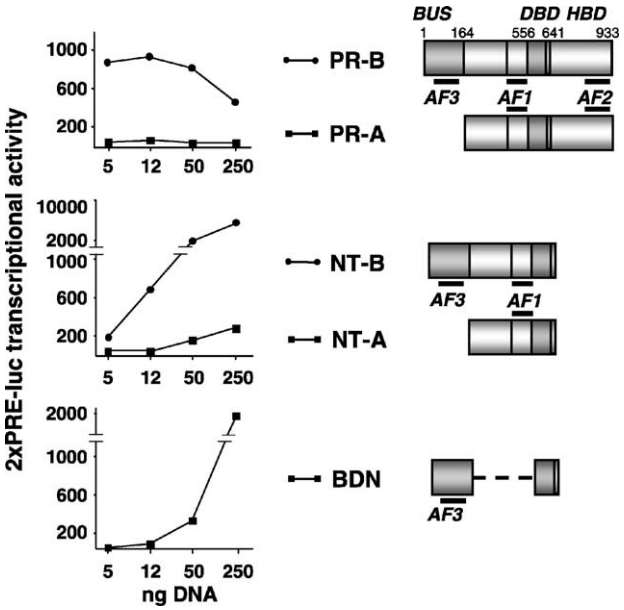


Fig. 1. AF3 synergizes with AF1 and AF2 at physiological receptor concentrations. Shown is a direct comparison of transcriptional activities of transiently transfected PR-B, PR-A, NT-B, NT-A, and BDN. PR-B contains AF3/AF1/AF2; PR-A contains AF1/AF2; NT-B contains AF3/AF1; NT-A contains AF1; and BDN contains AF3. BUS is the B-upstream segment consisting of the far N-terminal 164 residues of PR-B. DBD is the DNA binding domain between residues 556–641. HBD is the hormone binding domain between residues 641–933. HeLa cells were transiently transfected with 10–500 ng of cDNA and 1 μg PRE_2-TATA luciferase reporter plasmid. Transfections with full-length PR-B and PR-A were treated with 10 nM R5020. The HBD deleted constructs are constitutively active. For full-length receptors, transcriptional activity represents the difference between hormone-induced and basal (no hormone) levels. Basal activity is less than 5% of the corresponding values obtained with hormone. Data are plotted as firefly luciferase units normalized to *Renilla* luciferase internal control. Shown are representative experiments performed at least three times. Each point is the average of duplicate samples.

promoter containing tandem progesterone response elements (PRE_2-TATA luciferase). We tested constructs that expressed AF1 alone (NT-A), and AF1 + AF2 (PR-A). Both constructs elicited very low transcriptional activity at cDNA doses between 5 and 250 ng. Additionally, a C-terminal construct containing only AF2 that consisted of the DBD, hinge and HBD, also had little activity in this assay system (not shown). When BUS/AF3 was fused to NT-A and PR-A, producing NT-B and PR-B, respectively, a strong synergistic increase in activity was observed at all doses. Even BUS alone fused to the DBD had substantial activity, but only at the highest cDNA concentration (250 ng) tested. We conclude that at lower concentrations, AF3 has little intrinsic activity but synergizes strongly with AF1 and AF2 either alone or together. At higher concentrations, intrinsic AF3 activity increases to high levels and PR-B exhibits self-squelching which obscures the strong synergism.

We had previously analyzed a series of BUS deletion mutants, as well as random and site-directed BUS mutants in an effort to precisely map the AF3 domain [3]. These studies identified a core AF3 domain between residues 54–154 and three specific sites within this core that defined AF3 activity. These sites include ^{140}Trp and two LXXLL motifs designated L1 and L2. As shown in Fig. 2, mutation of L1, L2 or ^{140}Trp reduced transcriptional activity on a PRE_2-TATA luciferase promoter–reporter by 80% or more. Combined mutation of L1 and L2 sites reduced activity by 90%, to a level approximately equivalent to the very low activity of PR-A on the PRE_2-TATA promoter. These data suggest that inactivation of AF3-related activity can produce a receptor with a transcriptional phenotype resembling that of PR-A.

Our previous deletion mutagenesis studies had mapped a repressor region within the first 25 residues of BUS. Using a PCR-based random mutagenesis approach, we have now identified two highly hydrophilic residues, ^{5}Lys and ^{7}Lys, that produce a greater than 10-fold increase in transcriptional activity when mutated to a highly hydrophobic Trp residue. Inactivation of AF3 by mutating L1, L2 and ^{140}Trp completely prevented this activity increase, suggesting that the repressor function in BUS is linked to AF3-dependent transcriptional activity (not shown). Finally, the losses of activity with mL1, mL2 and $W^{140}A$ and gains of activity with the $K^{5}W/K^{7}W$ mutants were identical in BDN (BUS linked directly to the DBD) and full-length PR-B backgrounds (not shown), suggesting that these alterations of BUS functional domains affected both intrinsic and synergistic properties of AF3 activity.

3.2. The SUMO-1 binding motif is responsible for both autoinhibitory and transrepressor properties of PR

The autoinhibitory property of PR has been defined as the increased transcriptional activity seen upon deletion of a large region (IF) common to PR-A and PR-B located N-terminal to AF1 [9]. Recently, studies by Giangrande et al. [10], Huse et al. [11] and our laboratory [4] mapped this autoinhibitory function to subregions within IF. Within a subregion of IF containing the autoinhibitory function (residues 375–397), we identified a SUMO-1 consensus binding motif, ^{387}IKEE [17]. When a point mutation was introduced into this motif (^{387}IREE) in PR-B and PR-A, greater than 10-fold (PR-B) and 6–7-fold (PR-A) increases in transcriptional activity were observed from a PRE_2-TATA luciferase promoter–reporter (Fig. 3A). The fact that the PR-B $K^{388}R$ mutant remains a much stronger activator than the corresponding PR-A $K^{388}R$ mutant indicates that the intact SUMO-1 binding motif in PR-B tightly controls the strong AF3 synergism with AF1 and AF2. Interestingly, $K^{388}R$ mutant-dependent increases in transcriptional activity are hormone-dependent and completely absent in NT-A and NT-B constructs lacking the HBD [4]. We have also shown that the $K^{388}R$ mutation results in a complete loss of covalent attachment of the SUMO-1 protein to PR-A and PR-B, which is consistent with a single SUMO-1 binding motif present in the PR protein [4].

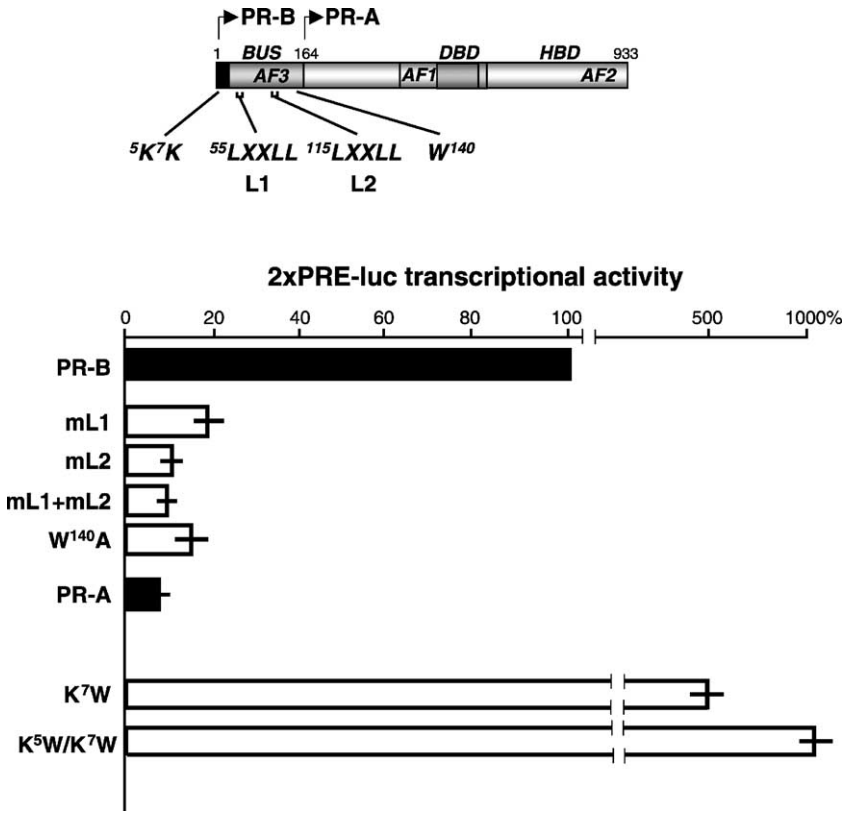


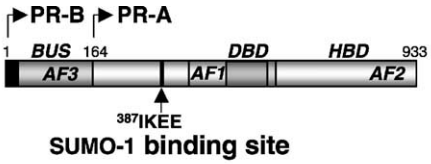
Fig. 2. Mapping critical BUS residues that define AF3 and the far N-terminal repressor region. Residues critical for AF3 activity were mutated as follows: L1 (^{55}LXXLL) mutated to mL1 (^{55}AXXAA); L2 (^{115}LXXLL) mutated to mL2 (^{115}AXXAA); and ^{140}W mutated to ^{140}A. Residues critical for repressor activity within residues 1–25 were mutated as follows: ^{5}K^{7}K mutated to ^{5}W^{7}W. Full-length wild type and BUS mutant receptors were transiently transfected into HeLa cells with 100 ng of cDNA and 1 μg PRE$_2$-TATA luciferase reporter plasmid. Transfection plates were treated with 10 nM R5020. Data are plotted relative to wild-type PR-B activity treated with R5020 set at 100%. All values were corrected for basal activity as described in Fig. 1. Basal activity in the absence of hormone is less than 5% of values obtained with hormone. Error bars represent the S.E.M. of at least three experiments.

PR also transrepress ERα as well as other steroid receptors, which may have physiological relevance in tissues coexpressing multiple steroid receptors. Fig. 3B shows the estradiol-induced activation of an ERE$_2$-TATA luciferase promoter–reporter by ERα. Cotransfection of PR-A and treatment with the progestin agonist R5020 results in the strong transrepression of estradiol-induced ERα activity. This PR-A-dependent transrepressor activity was completely abrogated by the K^{388}R mutant. Similar results were obtained with PR-B and its corresponding K^{388}R mutant except that PR-B are somewhat weaker transrepressors than PR-A (not shown). Analogous to properties governing autoinhibition of PR, the transrepressor effect of PR-A is entirely dependent upon the presence of a liganded HBD, and is therefore absent in NT-A and NT-B.

3.3. Conservation and physical properties of N-terminal activator and repressor sites

Fig. 4A shows the alignment of the human BUS region sequence to other mammalian (rabbit, horse, rat, mouse) and avian (chicken) sequences. It is clear that within the core AF3 domain (residues 54–154), only sequences containing and immediately surrounding L1, L2 and ^{140}Trp are uniformly conserved throughout these species. Additionally, ^{7}Lys, which produces a greater than 10-fold increase in PR-B activity when mutated with ^{5}Lys, is among the few residues fully conserved within the far N-terminal repressor region of BUS. As shown in Fig. 4B, the SUMO-1 binding motif is also conserved throughout the PR of mammalian and avian species. Our observations are consistent with the possibility that these conserved residues within the PR N-terminal region are integral parts of structural features fundamental to PR function.

Fig. 5 is a hydrophobicity plot of the BUS region showing that residues defining AF3 activity and synergism, including L1, L2 and ^{140}Trp, are contained within the major hydrophobic peaks of this region. The hydrophilic peak between residues 63–79 did not appear to have a major impact upon AF3-related activation since mutation of ^{69}Pro, ^{70}Ser and the conserved ^{77}Gln/^{78}Gln residues had little effect on PR-B induced transcription [3]. These data are consistent with those reported for AF1 of glucocorticoid receptors (GR) in which N-terminal hydrophobic residues were found to be more critical for transcription activation than hydrophilic residues. The K^{5}W/K^{7}W mutant, which relieves



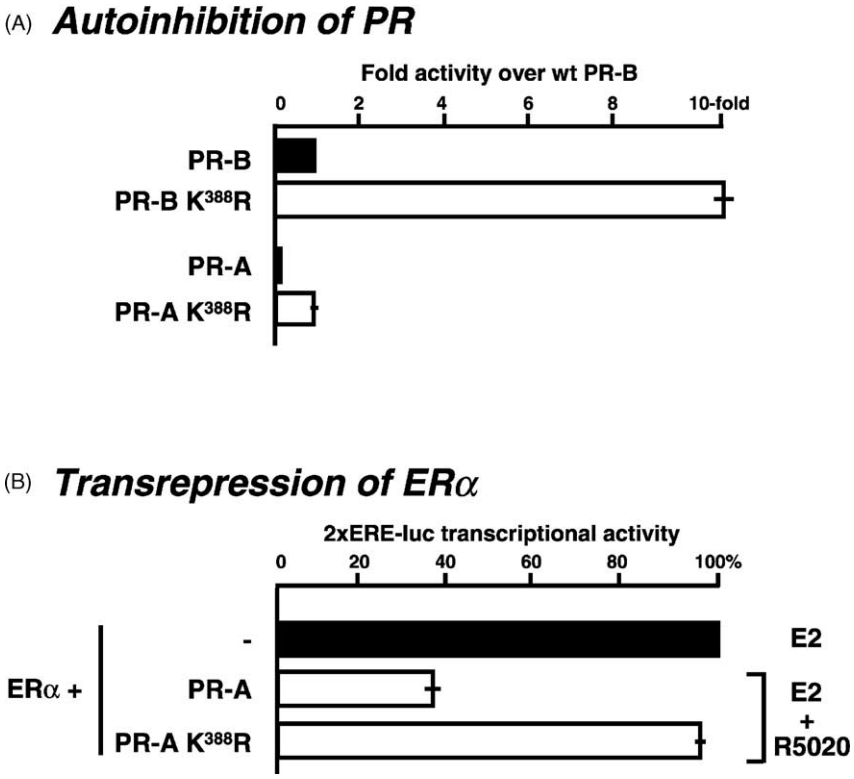


Fig. 3. Autoinhibition and transrepressor functions of PR require the SUMO-1 binding motif. HeLa cells were transiently transfected with 100 ng of cDNA and 1 μg PRE_2-TATA luciferase reporter plasmid. (A) Plates transfected with either wild-type (PR-B, PR-A) or mutant (PR-A $K^{388}R$, PR-B $K^{388}R$) cDNAs were treated with 10 nM R5020 and corrected for basal activity as described in Fig. 1. Activity is expressed as fold change as compared to wild-type PR-B. (B) All plates were transfected with 50 ng ERα cDNA alone and treated with 10 nM estradiol (E2) or ERα plus 50 ng PR-A or the PR-A $K^{388}R$ mutant cDNAs and treated with 10 nM E2 plus 10 nM R5020. Estrogenic activity is expressed as a percentage of ERα alone set at 100%.

the repressor function in the first 25 residues of BUS, resides within the major hydrophilic peak of this repressor region.

3.4. AF3 activity is DBD context-dependent

Fig. 6 shows transcriptional activities of AF3 in BDN and BDN chimeras that were tested on a PRE_2-TATA luciferase promoter–reporter. The chimeras were constructed by swapping either the PR DBD core region with the corresponding GR DBD core, or the PR DBD flanking regions with the corresponding GR DBD flanking sequences. The core region is defined by the DBD fragment that was used to obtain a high resolution NMR solution structure [24], and the limits of the flanking sequences were defined by the extended region originally demarcated as the "C" region of the full-length receptors plus four additional C-terminal residues comprising the PR nuclear localization signal [25]. Swapping the core PR DBD for the core GR DBD had no effect on transcriptional activity. However, swapping the core and flanking sequences of the core PR DBD for the GR DBD reduced activity by 70–75%. Both the N- and C-terminal flanking sequences of GR contributed to this reduction in activity (not shown). Sequence identity between the PR and GR DBD core is 90%, whereas it is 20–40% in the flanking regions. These data suggest that PR sequences in the DBD flanking regions are necessary for AF3 activity. Moreover, from unpublished studies in our laboratory, we know that substituting the PR DBD with heterologous DBDs such as the GAL4 DBD or the ERα DBD also results in a substantial loss of AF3 activity. Taken together, these observations support the hypothesis that expression of AF3-related functional activities can be allosterically regulated by specific

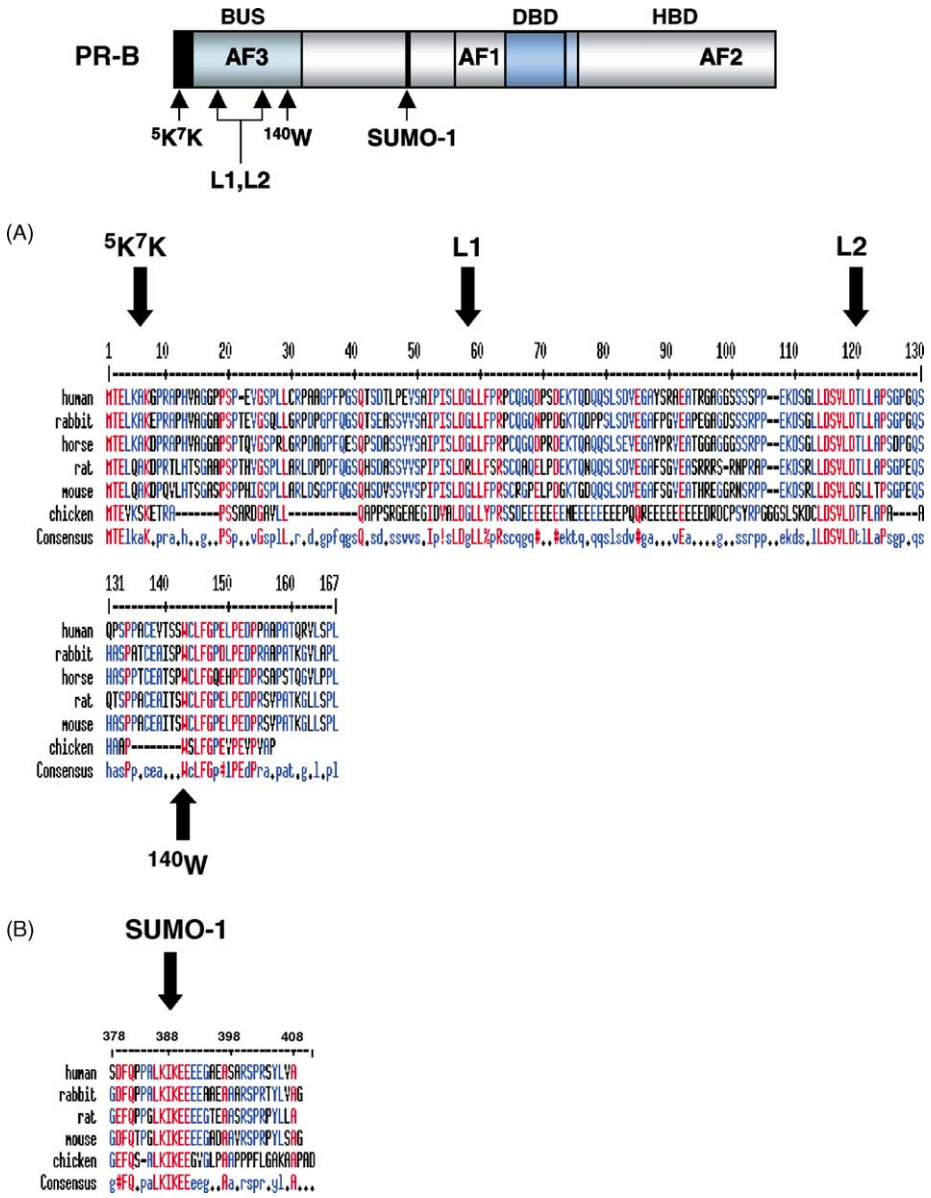


Fig. 4. Residues responsible for AF3 activator and N-terminal repressor properties of PR are highly conserved. Sequence alignment of various species shown for the BUS (A) and SUMO-1 binding (B) regions. Conserved residues throughout the species listed are shown in red in the consensus sequence and include residues comprising ^{5}K^{7}K, L1 (^{55}LXXLL), L2 (^{115}LXXLL), ^{140}W (A); and the SUMO-1 binding motif (^{387}IKEE) (B).

amino acid residues within the DBD region, and, therefore, upon recognition of context-specific DNA response element sequences at a gene promoter. Clearly for PR, and probably for other transcription factors, analysis of their AFs through use of heterologous DBDs may yield skewed information.

3.5. Biochemical and biophysical studies provide a mechanistic basis for explaining differences in N-terminal function

In recent studies using highly purified NT-B and NT-A, we performed biochemical and biophysical analyses that yielded the results summarized in Table 1 [5,6]. First, NT-A and NT-B purified to greater than 95% homogeneity were transcriptionally competent in a cell-free in vitro transcription assay. Nitrocellulose filter binding studies indicated that both NT-A and NT-B bind cooperatively, and with similar affinities, to a single PRE oligonucleotide whose sequence was obtained from the GRE/PRE of the tyrosine aminotransferase gene promoter [38]. The DBD alone, however, bound weakly with a 10-fold lower affinity than NT-A and NT-B (not shown) suggesting that N-terminal regions common to both PR-A and PR-B are responsible together with the DBD, for cooperative interactions between the receptors and their cognate response elements. By sedimentation equilibrium analysis, NT-A and NT-B were monomeric in

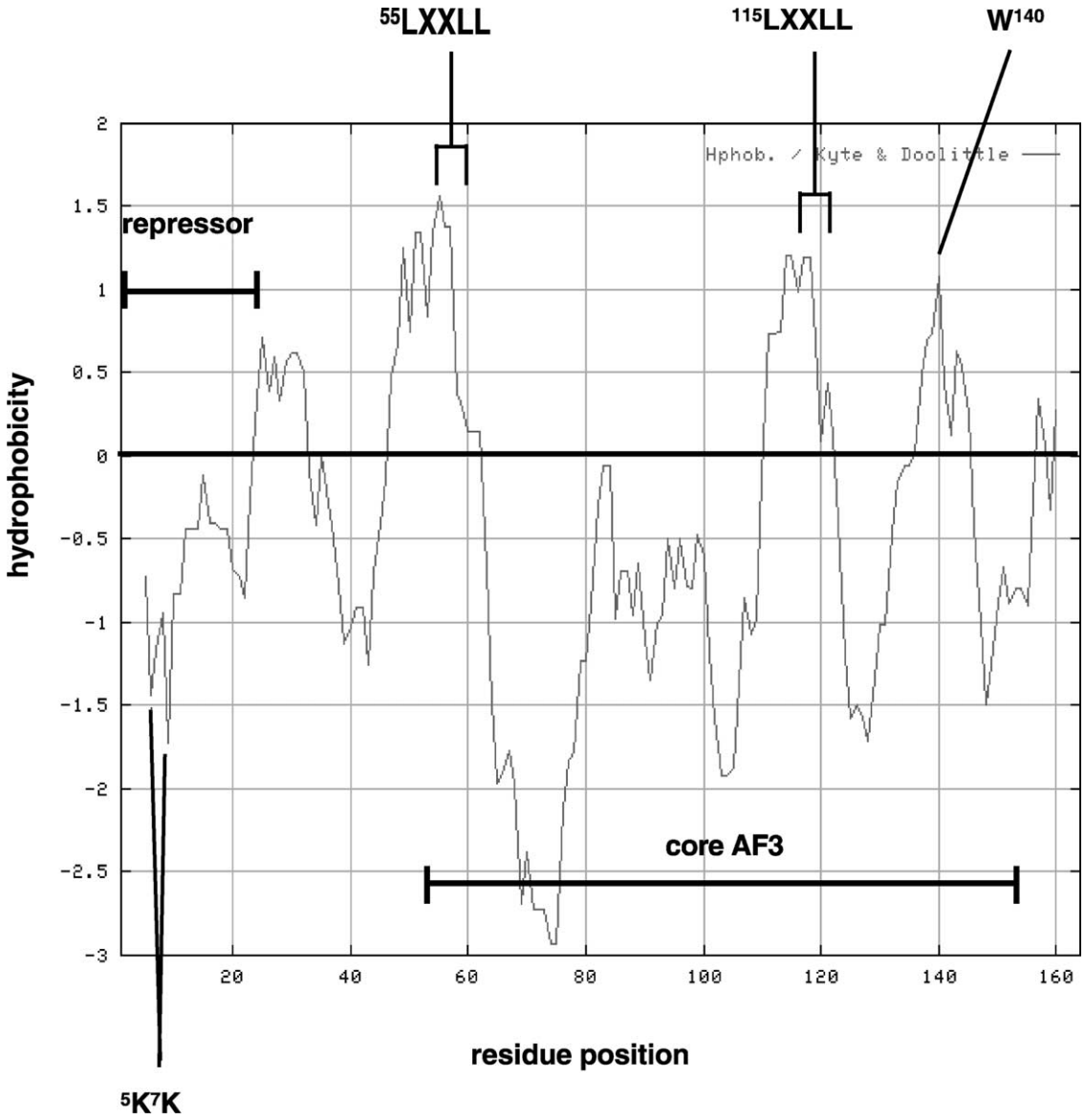
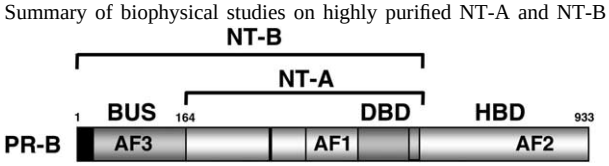


Fig. 5. Hydrophobicity plot of the BUS region. Shown are the repressor (residues 1–25) and AF3 core (residues 54–154) in BUS as well as the residues within these regions that define their respective functional properties.

solution suggesting that N-terminal mediated assembly does require DNA binding. These data do not speak to the role of the HBD, if any, in mediating dimerization in solution. Furthermore, limited proteolysis studies indicated that the structure of NT-A and NT-B are similarly ordered, with regions of higher stability tending to be bordered by known phosphorylation sites or defined by the previously mapped AF1 domain. Further stabilization of sequences around and within AF1, and the hinge region, was observed upon binding to a PRE. Interestingly, while NT-A and NT-B exhibited ordered structure, N-terminal proteolytic fragments lacking the DBD were rapidly degraded suggesting that the N-terminal region was largely unstructured in the absence of the DBD. We conclude that the DBD is involved in nucleating or stabilizing the structure of the N-terminal region. The BUS region was rapidly degraded even in NT-B and appeared to possess little intrinsic structure under any condition.

Sedimentation velocity experiments indicated that NT-A and NT-B are elongated and rod-like in shape with NT-B being more highly elongated than NT-A due to the presence of BUS in an extended conformation. More importantly, an integral distribution plot of the sedimentation velocity data show, counter-intuitively, that NT-A are more structurally heterogeneous (12%) than NT-B (4%). Thus, in NT-B, BUS limits the number of different structural transitions traversed by the N-terminal region in solution. These results are consistent with the hypothesis that two major structural features impact N-terminal function: first, DNA-induced allostery transduced through the DBD stabilizes N-terminal structure;

Table 1
Summary of biophysical studies on highly purified NT-A and NT-B polypeptides



	NT-A	NT-B
Purification	>95	>95
In vitro transcription (fold over basal)	20X	20X
DNA binding (DBD alone, weak)	Cooperative	Cooperative
Sedimentation equilibrium	Monomer: 59 kDa	Monomer: 71 kDa
Proteolytic mapping w and w/o PRE	Ordered structure (+DBD)	Ordered structure (+DBD) except BUS
Sedimentation velocity	Prolate ellipsoid (9:1) Heterogenous: ΔS20,w 12%	Prolate ellipsoid (20:1) Homogenous: ΔS20,w 4%

For a detailed description of methods and data analyses, see Bain et al. [5,6].

and second, that BUS minimizes the ensemble distribution of conformers adopted by NT-B. Together these two elements provide a mechanistic basis for the involvement of N-terminal regions in transcription activation of full-length PR and for functional differences between PR-B and PR-A.

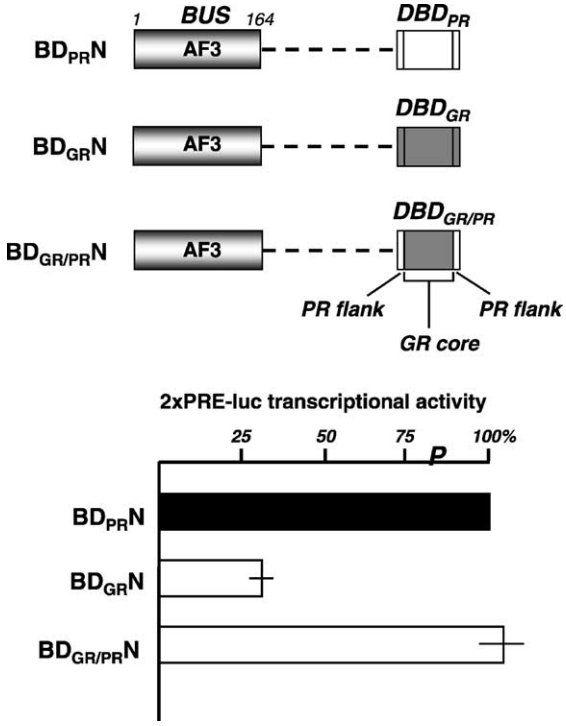


Fig. 6. DNA binding domain context dependence of AF3-dependent transcriptional activity. The $BD_{PR}N$ construct is composed of BUS (residues 1–164) fused to the PR DBD (residues 556–645); $BD_{GR}N$ is BUS fused to the human GR DBD (residues 430–519); $BD_{GR/PR}N$ is BUS fused to the human GR core DBD (residues 441–506) flanked by PR residues 556–565 at the N-terminus and PR residues 632–645 at the C-terminus. HeLa cells were transiently transfected with 100 ng of cDNA and 1 μg PRE_2-TATA luciferase reporter plasmid. Activity is expressed as a percentage of $BD_{PR}N$ set at 100%.

4. Discussion

The importance of N-terminal functional domains in steroid receptor action is becoming increasingly apparent. For example, with the androgen receptors (AR), AF1 in the N-terminal region predominates in determining the overall activity of the receptors [26]. Additionally, N- and C-terminal interactions have been well-documented for AR and PR and a growing number of coregulators are known to interact with N-terminal regions of nuclear receptors and/or regulate their activities [27–30,35]. PR, comprised of A and B isoforms, provide an ideal model system to probe the mechanistic details by which receptor N-terminal regions modulate function. PR-A and PR-B are identical in sequence except for 164 amino acids at the far N-terminus of PR-B that contains AF3, and thus the extensive isoform-specific functional differences are, in principle, physically linked solely to this region. Our results in the present studies precisely map activator and repressor sites in the N-terminal regions of PR and suggest that AF3, by synergizing with AF1 and AF2, produces a generally stronger transcriptional phenotype in PR-B than PR-A. Further, our recent biochemical and biophysical studies on purified N-terminal fragments point to possible mechanisms by which the strong transcriptional phenotype of PR-B might occur.

4.1. AF3 synergism with AF1 and AF2 is controlled by the SUMO-1 binding motif

When AF3 or AF1 is assayed in isolation using the BDN (AF3) or NT-A (AF1) constructs, the resulting transcriptional activities were low to absent at cDNA doses less than 50 ng. These doses generate amounts of receptor proteins resembling physiological levels. However, when AF3 and AF1 are present together as in NT-B, a synergistic increase in activity is seen at concentrations below 50 ng. Above 50 ng BDN activity increases to levels approaching that of NT-B. Thus, AF3 can exhibit strong transcriptional activity, but only at high protein concentrations. Parenthetically,

these data point to the importance of understanding receptor concentrations when evaluating transcriptional activities in vitro. AF3 synergizes with AF1 and AF2 when both are present as seen by comparing the activities of PR-B (AF3, AF1, AF2) with PR-A (AF1, AF2) and BDN (AF3) at doses less than 50 ng. Interestingly, this synergism is highly tempered by the presence of the SUMO-1 binding motif, ^{387}IKEE, located in the N-terminal region common to both PR-B and PR-A. When this motif is mutated to ^{387}IREE, the activity of PR-B increases by greater than 10-fold, whereas the activity of NT-B is unchanged. These findings suggest that at intracellular receptor levels that more closely resemble physiological, the potential for AF3 to synergize with AF1 and AF2 is tightly controlled. Under conditions where PR-B are not sumoylated or under-sumoylated, extremely high levels of synergism-dependent activation could in theory be achieved. This could represent a mechanism whereby gene expression levels could be rapidly turned up or down. Synergism between AF1 and AF2 is also controlled by sumoylation. However, in the absence of AF3 the maximum potential for AF synergism is a fraction of that seen with non-sumoylated PR-B.

As with AR, the activities of PR N-terminal constructs such as NT-B and BDN at higher doses make a more pronounced contribution to the transcriptional phenotype of PR-B than the C-terminal HBD region containing AF2, which has little intrinsic activity on promoters examined so far [26]. However, our results with the K^{388}R SUMO-1 binding mutant suggest that maximal AF synergy is intimately dependent upon C-terminal sequences within a liganded HBD. Additionally, recent unpublished studies in our laboratory demonstrate that AF3 related synergy requires multiple tandem PREs and is inoperative on a synthetic minimal promoter construct with a single palindromic response element. It is conceivable that the arrangement, type and number of heterologous *cis*-acting promoter elements adjacent to a single PRE may play a critical role in the expression of AF3-related synergy on endogneous promoters.

4.2. Repressor properties of PR are related to synergy control

We have presented compelling evidence that the autoinhibitory properties of PR-A and PR-B are intimately linked to the SUMO-1 binding or "synergy control" motif [18]. Interestingly, when we tested the ability of PR to transrepress ERα, we obtained similar results. Both PR-A, and to a lesser extent PR-B, inhibit the transcriptional activity of estradiol-induced ERα on an ERE$_2$-TATA luciferase promoter–reporter. This effect requires the liganded HBD of PR and an intact SUMO-1 binding motif analogous to the requirements for synergy control in full-length receptors. It does not appear that this effect is due to squelching of limited coregulator pools since transrepression occurs at concentrations of PR well below the levels eliciting maximal transcriptional responses. Whether PR are able to bring tethered synergy control factors to the ERα-regulated promoter complex remains to be investigated.

A second repressor region in the far N-terminus of BUS was also identified. Conversion of hydrophilic ^{5}Lys and ^{7}Lys to hydrophobic Trp residues resulted in 10-fold increases in transcriptional activities of PR-B, comparable to those seen with the SUMO-1 binding mutant. However, this increased activity was not dependent upon a liganded HBD since BDN lacking an HBD exhibited the same increased activity as PR-B. Since the increased activity was abrogated by mutation of L1, L2 and ^{140}Trp in BUS (data not shown), the mechanism of repression may be more directly linked to the overall physical properties of AF3 and BUS.

4.3. A model for N-terminal linked activation

We propose the model shown in Fig. 7 based upon mechanistic insights provided by biochemical and biophysical analyses and novel functional properties of the PR N-terminal regions. As shown in part A, the regions of PR-A and PR-B N-terminal to the DBD are largely unstructured, with BUS assuming an extended, non-globular conformation. Ordered structure (part B) is induced by the presence of the DBD, and further stabilization is achieved, particularly for sequences around and within AF1 and the hinge domains (blue), upon binding to DNA. The intrinsic as well as synergistic transcriptional activities attributed to AF3 in BUS is DBD context-dependent and therefore is intimately linked to the number and specific sequence of response elements. The LXXLL motifs (L1, L2) as well as ^{140}Trp in BUS define AF3-related activity and may mediate intra- or intermolecular interactions. The unusual presence of two LXXLL motifs in BUS (motifs that are more commonly found on coactivators) suggest that BUS is involved in protein–protein interactions that remain to be defined. We view BUS as a "tethered coregulator" and predict that other transcription factors may have similar domains. Finally, as shown in part B, the greater heterogeneity seen with the ensemble distribution of NT-A (12%) as compared to NT-B (4%) conformers leads us to propose that, mole for mole, a higher proportion of NT-B molecules exist in an active (✱) conformation resulting in the generally stronger transcriptional phenotype of this isoform.

Fig. 7C illustrates the critical role of the liganded HBD in the tight control of potentially high levels of AF synergism in PR-A (✱) and particularly AF3-dependent synergism in PR-B (✱✱✱), as seen by the effects of desumoylation of the full-length receptors. This figure illustrates the likelihood that the N-terminal region, particularly BUS, and the C-terminal HBD participate in critical intramolecular interactions that minimize the ensemble distribution of receptors to a conformation(s) eliciting optimal AF synergism. Lastly, this figure does not address the additional structural complexity imparted by intermolecular interactions between

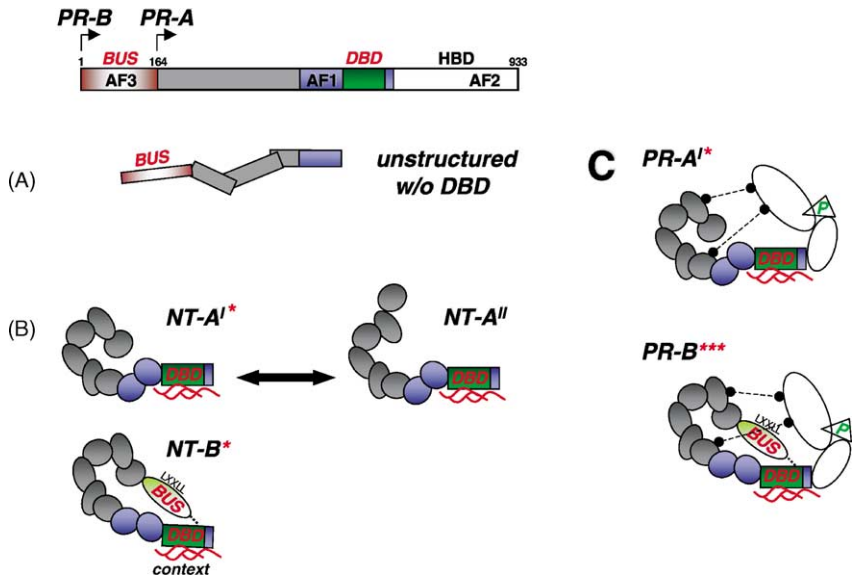


Fig. 7. DNA binding influences the PR N-terminal region and liganded HBD is required for maximal AF synergism. (A) Without the DBD, the N-terminal region is unstructured with BUS present in an extended conformation. (B) Addition of the DBD stabilizes the N-terminal region in a structurally definable conformation. Between BUS and the DBD, NT-A and NT-B are structurally similar. However, NT-A exhibits greater heterogeneity of molecularly active (*) plus inactive conformers (NT-A^{I}/NT-A^{II}) than NT-B. We propose that NT-B is enriched in the active (*) population of conformers, and possibly, that they are stabilized by BUS. The LXXLL motifs (L1, L2) in BUS may mediate intramolecular communication (---) or intermolecular interactions within or between dimers or with coregulatory proteins, to induce the greater stability of NT-B. BUS (AF3)-dependent activation is modified by the DBD context and, therefore, by the composition of the corresponding DNA response element sequences to which the receptors are bound. (C) Maximal AF synergism is achieved only with the liganded HBD present, which implies communication between N- and C-terminal regions (---) to stabilize the most active population of PR conformers (***). We predict that protein–protein interactions in addition to DNA-induced allostery influence the structure of the classical subdomains (DBD and HBD) within the full-length receptors.

receptor dimers and between receptors and coregulatory proteins, although it does imply that the structural basis required for these higher level interactions resides in the subset of conformers that are stabilized in the liganded full-length receptors.

Nuclear receptors were initially characterized as complex molecules having modular structure and distinct domains that can function independently. These properties have justified using isolated domains (e.g. core DBD and HBD) for high resolution structural analyses. However, we believe that such analyses yield a static and therefore incomplete structural picture. Based on our studies with PR [5,6,32] as well as those of others on GR [28,31,33,34] and AR [2,36], the emerging structural model of full-length receptors depicts an N-terminal region that communicates allosterically and through intramolecular interactions with the DBD and HBD in order to stabilize active conformers and optimize binding with coregulatory proteins. Our studies with purified NT-A and NT-B [5,6], and those performed by Williams and Sigler with purified PR HBD [37] clearly indicate that N- and C-terminal receptor regions, when expressed independently, are purely monomeric in solution. Since purified, full-length PR-B does undergo self-assembly to dimers in solution (D. Bain, personal communication), the relationship between structure and function of the DBD and HBD "domains" will likely only be understood in the context of the full-length receptors.

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