Mapping the Unique Activation Function 3 in the Progesterone B-receptor Upstream Segment

TWO LXXLL MOTIFS AND A TRYPHTHAN RESIDUE ARE REQUIRED FOR ACTIVITY

Received for publication, July 19, 2001, and in revised form, August 20, 2001
Published, JBC Papers in Press, August 22, 2001, DOI 10.1074/jbc.M106843200

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Progesterone receptors (PR) contain three activation functions (AFs) that together define the extent to which they regulate transcription. AF1 and AF2 are common to the two isoforms of PR, PR-A and PR-B, whereas AF3 lies within the N-terminal 164 amino acids unique to PR-B, termed the "B-upstream segment" (BUS). To define the BUS regions that contribute to AF3 function, we generated a series of deletion and amino acid substitution mutants and tested them in three backgrounds as follows: BUS alone fused to the PR DNA binding domain (BUS-DBD), the entire PR-B N terminus linked to its DBD (NT-B), and full-length PR-B. Analyses of these mutants identified two regions in BUS whose loss reduces AF3 activity by more than 90%. These are associated with amino acids 54–90 (R1) and 120–154 (R2). R1 contains a consensus LXXLL motif (L1) identical to ones found in nuclear receptor co-activators. R2 is adjacent to a second nuclear receptor box (L2) at 115LXXLL119 and contains a conserved tryptophan (Trp-140). Their mutation completely disrupts AF3 activity in a promoter and cell type-independent manner. Critical mutations elicited similar effects on all three B-receptor backgrounds. This underscores the probability that these mutations alter a process linking BUS structure to the function of full-length PR-B in a fundamental way.

The nuclear receptor (NR) family of proteins share several functional domains, including highly conserved, centrally located DNA binding domains (DBD) and conserved C-terminal hormone binding domains (HBD) that contain ligand-binding and co-activator-binding sites. The ligand-activated receptors bind DNA and regulate gene transcription through regions called activation functions (AFs), AF1 in the N-terminal region upstream of the DBD and AF2 in the HBD. These AFs are thought to regulate transcription directly, by contacting the basal transcription machinery, or indirectly, by binding co-regulatory proteins. Indeed, the latter appears to be a major mechanism, and a large number of receptor co-activators and co-repressors have been identified (1–3). Co-activators bind to the HBD through NR boxes containing consensus LXXLL motifs (4).

The effects of the steroid hormone, progesterone, are mediated through nuclear progesterone receptors (PR). They exist naturally as two isoforms and are transcribed from two promoters present on a single gene (5). Full-length human PR-B are 933 amino acids in length. Full-length human PR-A are only 769 amino acids in length because they lack 164 N-terminal amino acids present in PR-B. The extra 164 amino acids in the PR-B upstream segment (BUS) are responsible for important functional differences between the two PR isoforms, because BUS contains a strong third AF (AF3) (6). Two additional AFs, analogous to those of other NR, are shared by PR-A and PR-B; AF1 maps to 91 amino acids just upstream of the DBD and AF2 lies within the HBD (7, 8). On most promoters and cell types, PR-B are much stronger transactivators than PR-A (6, 8, 9), presumably due to the influence of AF3. However, there are important exceptions (9), as shown by cDNA microarray studies that identify endogenous genes in breast cancer cells uniquely up-regulated by PR-A.2 There are other interesting differences between the two PR isoforms as defined by in vitro studies. When bound by the antiprogestin RU486, only PR-B have partial agonist activity (7, 10, 11), whereas only PR-A are dominant inhibitors of transcription by PR-B and other steroid receptors (9, 12–14). Studies in vivo involving PR null mice, and transgenic mice overexpressing one of the two PR isoforms, confirm that the two PR serve different physiological functions (15–18). There are wide fluctuations in PR-A:PR-B ratios in female reproductive tissues during the normal menstrual cycle (19, 20), and abnormal PR-A:PR-B ratios are associated with highly malignant forms of reproductive tract cancers (21). The PR-A to PR-B ratio also varies widely among human breast cancers, with unknown consequences (22).

Although the transcriptional differences between PR-A and PR-B have been soundly documented, the mechanisms underlying these differences remain unknown. We have postulated that AF3 in BUS plays an important role, and we have compared the structures of N-terminal A (NT-A) and N-terminal B (NT-B), which are constitutively active forms of PR lacking only the HBD of the full-length receptors (23, 24), in an effort to explain their functional differences. These studies indicate that the N termini exist in a non-globular, extended conforma-

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1 The abbreviations used are: NR, nuclear receptor; DBD, DNA binding domain; HBD, hormone binding domain; AF, activation function; PR, progesterone receptor; BUS, PR-B upstream segment; NT-A, N-terminal region of PR-A; NT-B, N-terminal region of PR-B; R1, region 1 of BUS; R2, region 2 of BUS; NLS, nuclear localization signal; GR, glucocorticoid receptor; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; MMTV, mouse mammary tumor virus.

2 J. K. Richer and K. B. Horwitz, submitted for publication.

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tion. Interestingly, BUS is even more extended than the downstream regions common to NT-A and NT-B; it is not an independent structural domain. Rather, the ensemble distribution of conformational states is more restricted in NT-B than in NT-A. This suggests that in NT-B BUS stabilizes the rest of the N terminus in a functionally active set of conformations. We therefore attempt to explain the mechanisms by which BUS exerts this stabilizing effect to yield AF3 activity.

To that end, we have now mapped BUS in detail, by generating a series of deletion and amino acid substitution mutants. We found two BUS subregions, designated R1 (residues 54–84) and R2 (residues 120–154), that are necessary for efficient transcription by PR-B. Removal of either of these regions decreases the transcriptional efficiency of PR-B by >60%. Removal of both reduces their transcriptional efficiency by >90%. These regions define hydrophilic or hydrophobic peaks within BUS. Mutation of key amino acids within these peaks leads to major decrements in function. Two sites contain canonical LXXLL motifs, usually found on NR co-activators, and are required for their binding to the HBD (4, 25). When these are mutated in BUS, AF3 activity is lost. Tryptophan 140 is also absolutely required for AF3 activity. We discuss the implications of this for BUS structure, and we speculate that BUS is involved in protein-protein interactions that generate AF3 function.

EXPERIMENTAL PROCEDURES

Recombinant Plasmids and Site-directed Mutagenesis—Wild-type pSG5-BUS-DBD, which expresses BUS (amino acids 1–164) of human PR-B, fused to the human PR DBD and nuclear localization signal (NLS, amino acids 556–645), was constructed by removing BUS from the full-length PR-B expression vector (pSG5-hPR1) as described previously (6). BUS deletion mutants were initially constructed in pSG5-BUS-DBD by removing wild-type BUS with EcoRI and RevII restriction enzymes and replacing it with PCR-generated deletion BUS mutants. Site-directed mutagenesis was carried out by PCR amplification of wild-type BUS using oligonucleotides containing specific point mutations. Mutant constructs were used to replace wild-type BUS at the EcoRI/RevII sites in the original vector. All mutations were verified by automated fluorescence sequencing (University of Colorado Health Sciences Center, Core Lab). All BUS deletions and mutants were subsequently moved by direct subcloning, first into pSG5-NT-B, which expresses the entire PR-B N terminus, plus the DBD and NLS (amino acids 1–645), and lacks only the HBD (6), and second into full-length PR-B (pSG5-hPR1). Correct expression of all constructs was verified by transient transfection into COS cells and immunoblotting.

Transfection and Immunoblotting—Expression of wild-type and mutant PR constructs was verified in COS monkey kidney cells. Briefly, cells were plated at 3 × 10^5 per 100-mm plate 1 day prior to transfection. Two μg of expression plasmid and 3 μg of internal control plasmid (β-galactosidase) were transiently transfected into each dish by calcium phosphate co-precipitation (26). Cells were collected and digested in Elution Buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 0.1% bovine serum albumin) containing a protease inhibitor mixture (Roche Molecular Biochemicals); extracts were normalized to β-galactosidase activity and resolved by SDS-PAGE (12% acrylamide for pSG5-BUS-DBD, 10% for pSG5-NT-B, and 7.5% for pSG5-PR-B). Immunoblots were either probed with a polyclonal antibody to the PR DBD (gift of David Toft, Mayo Medical School, Rochester, MN) (27) or with anti-PR monoclonal antibodies AB-52 and B-30 (28). Bands were detected by enhanced chemiluminescence.

Transcription Assays—HeLa cervical carcinoma cells were plated in minimum Eagle’s medium containing 5% fetal bovine serum (twice charcoal-stripped fetal bovine serum for experiments containing full-length PR-B constructs), at a density of 1.1 × 10^6 cells per 60-mm dish, 1 day prior to transfection. Cells were transfected by calcium phosphate co-precipitation (26) using concentrations of expression vectors ranging between 5 and 500 ng, depending on the experiment. Two reporters were tested each at 2 μg/dish as follows: a PRE–TATA-luciferase reporter containing two tandem copies of a perfect palindromic PRE, or a mouse mammary tumor virus (MMTV) promoter-luciferase construct (gift of S. Nordeen, University of Colorado, Denver, CO) (29). SV40–Renilla luciferase was added as an internal control vector at 20 ng per dish. After transfection, cells were grown for 24 h prior to collection.

experiments with full-length PR-B, the synthetic progesterin R5020 was added to each dish at a final concentration of 10 or 100 nM as indicated. The HBD truncated constructs are constitutively active. Cells were collected in 150 μl of lysis buffer (Promega), and 50 μl were analyzed on a dual luminometer. Results were normalized to Renilla luciferase activity, and expressed as a relative activity.

PR-negative T47D human breast cancer cells (30) were transfected by electroporation. Briefly, cells were collected and resuspended in minimum Eagle’s medium at 10 × 10^6 cells/ml. Cell suspensions were electroporated at 220 V, 950 microfarads with 1 μg of expression vector, 2 μg of luciferase reporter, and 250 ng of SV40–Renilla luciferase per 1 × 10^6 cells and plated in 35-mm dishes. After a recovery period of 16–20 h, cells were treated with 10 nM R5020 for an additional 24 h. Cells were collected in 100 μl lysis buffer (Promega), and 50 μl were read on a dual luminometer.

RESULTS

Transcription by the Wild-type BUS Constructs—The present study analyzes the activity of BUS, amino acids 1–164 of human PR-B, in three different background (Fig. 1A) as follows: BUS-DBD (amino acids 1–164 plus 556–645), which contains BUS linked directly upstream of the PR DBD and NLS; NT-B (amino acids 1–645), which contains the entire N terminus of PR-B including the DBD and NLS; and full-length PR-B (amino acids 1–933). The BUS near APD3 in BUS alone (BUS-DBD), together with AF1 (NT-B), or with AF1 + AF2 (PR-B). As a control, most studies also include the N terminus of PR-A (NT-A), which has only AF1, or full-length PR-A, which has AF1 + AF2 but not AF3. Note that constructs lacking the HBD are constitutively active. The activity of full-length PR-A and PR-B was tested using saturating concentrations of the synthetic progesterin, R5020.

Fig. 1B shows the transcriptional activity of these constructs expressed in HeLa cells after transfection with increasing concentrations of the cDNA expression vectors. The cells were also transfected with a PRE–TATA luciferase promoter-reporter, with Renilla luciferase to monitor transfection efficiency, and treated with or without R5020 as needed. Data are reported as relative luciferase activities of triplicate transfections, after correction for the Renilla control. Full-length PR-B characteristically have a bell-shaped dose-response curve, with maximal activity at intermediate protein concentrations, and decreasing activity at higher concentrations due to “squelching” (31). PR-A show a similar pattern of activity, but maximal transcription is generally less than 10% that of PR-B (6, 31). Unlike the full-length receptors, the dose-response of NT-B and BUS-DBD does not saturate, with increasing activity at increasing protein concentrations. Their maximal activity is equivalent to, or greater than, that of full-length PR-B. This pattern of transcription suggests that squelching requires the presence of the HBD. NT-A has lower activity, analogous to that of PR-A. Because of the variations in activity in varying cDNA levels, analysis of the BUS mutant constructs was always carried out by transfection of multiple cDNA concentrations. In some cases, only the maximal activity is reported.

Phosphoserine and Cysteine Residues Do Not Impact AF3

Transcription—PR are richly phosphorylated proteins (32–34). PR-B are more highly phosphorylated than PR-A because of multiple potential phosphorylation sites in BUS, among which phosphorylation of Ser-20, Ser-81, Ser-102, and Ser-162 have been documented in vivo (32, 35, 36). We previously mutated, individually or collectively, all of the potential BUS phosphorylation sites, and we found no decrement in the transcriptional activity of PR-B (Table I) (34). Additionally, there are several cysteine residues in BUS that could theoretically be involved in disulfide bond formation. These were mutated to alanine either singly or together. Transcriptional activity of the mutant PR-B constructs was analogous to that of wild-type PR-B (Table I and data not shown). We conclude that neither

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Deletions in BUS Identify Two Regions Critical for AF3 Function—To target sites important for AF3 activity, we generated a series of stepwise deletions from the N terminus, C terminus, and internal regions of BUS. This was accomplished by a PCR-based technique in which specific BUS regions were amplified and used to replace wild-type BUS in expression vectors. BUS deletion mutants were cloned into all three transcriptionally active BUS contexts: BUS-DBD (6), NT-B (14), and full-length PR-B. The accuracy of all constructs was verified by DNA sequencing and by protein expression following transient transfection. Fig. 2 shows a representative Western blot of N-terminal deletion constructs in the context of PR-B. Vectors containing PR-B deletions were transiently expressed in COS cells, and whole cell lysates were prepared and separated on a 7.5% SDS-PAGE gel. Proteins were transferred to nitrocellulose, and the blot was probed with an anti-PR monoclonal antibody whose epitope lies in the N-terminal region common to both PR isoforms (28). The 120-kDa full-length wild-type PR-B is shown in lane 1. Stepwise deletions of PR-B ranging from 23 to 148 amino acids are shown sequentially in lanes 2–7. The 94-kDa PR-A protein is shown in lane 8.

Fig. 3, A–C, shows the consequences of stepwise deletions from the BUS N terminus (A), C terminus (B), or both (C) on the transcriptional activity of BUS in the context of the following three backgrounds: BUS-DBD, NT-B, or PR-B. Expression vectors were transfected into HeLa cells at 500 (BUS-DBD and NT-B) or 25 ng/dish (PR-B) (see Fig. 1), and transcription was measured from the PRE2-TATA-luciferase reporter. All data points represent the average of at least four independent assays in a minimum of two separate experiments. The activity of wild-type constructs containing BUS, in each of the three backgrounds, was set at 100%. The data are summarized in Table II and include statistical analyses.

Stepwise deletions of BUS from the N terminus, ranging from ∆23 to ∆148 amino acids, in BUS-DBD, NT-B, and PR-B, are shown in Fig. 3A. The first deletion, ∆23, consistently increases (135–185%) receptor activity in all three backgrounds, compared with wild-type BUS constructs (100%). This suggests that the first 23 amino acids of BUS contain an inhibitory activity. Deletion of the first 54 amino acids (∆54) restores transcription to approximately wild-type, suggesting that AF3 activity does not reside in these residues. Note that when the three BUS backgrounds are compared, deletions always have the most deleterious effects in the context of BUS-DBD. We speculate that AF1 and/or AF2 in the other two contexts partially compensate for the loss of AF3. Deletion to ∆84 dramatically lowers (by 86%) the transcriptional activity of BUS-DBD and extensively lowers the activity of NT-B (by 64%) and PR-B (by 66%), suggesting that at least one important region of AF3 lies within amino acids 54–84 of BUS. Additional deletions to amino acids 114, 135, and 148 reduce the activity of BUS-DBD to negligible levels (below 5%) and also cause major decrements of function in NT-B and PR-B. The PR DBD alone has no activity.

**TABLE I**

| Mutation(s) | Number of amino acid changes |
|-------------|-----------------------------|
| S20A/S25A  | 2                           |
| S20A/S25A/S99A/S100A/S101A/S102A/S103A | 6 |
| S20A/S25A/S99A/S100A/S101A/S102A/S130A/S162A | 7 |
| S20A/S25A/S99A/S100A/S101A/S102A/S130A/S162A/S79A/S81A | 8 |
| C29A       | 1                           |
| C64A       | 1                           |
| C134A      | 1                           |
| C141A      | 1                           |
| C29A/C141A | 2                           |
| C134A/C141A | 2                           |

 phosphorylation nor disulfide bond formation are critical for the AF3 activity of BUS.

**Deletions in BUS Identify Two Regions Critical for AF3 Function**—To target sites important for AF3 activity, we generated a series of stepwise deletions from the N terminus, C terminus, and internal regions of BUS. This was accomplished by a PCR-based technique in which specific BUS regions were amplified and used to replace wild-type BUS in expression vectors. BUS deletion mutants were cloned into all three transcriptionally active BUS contexts: BUS-DBD (6), NT-B (14), and full-length PR-B. The accuracy of all constructs was verified by DNA sequencing and by protein expression following transient transfection. Fig. 2 shows a representative Western blot of N-terminal deletion constructs in the context of PR-B. Vectors containing PR-B deletions were transiently expressed in COS cells, and whole cell lysates were prepared and separated on a 7.5% SDS-PAGE gel. Proteins were transferred to nitrocellulose, and the blot was probed with an anti-PR monoclonal antibody whose epitope lies in the N-terminal region common to both PR isoforms (28). The 120-kDa full-length wild-type PR-B is shown in lane 1. Stepwise deletions of PR-B ranging from 23 to 148 amino acids are shown sequentially in lanes 2–7. The 94-kDa PR-A protein is shown in lane 8.

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| S20A/S25A/S99A/S100A/S101A/S102A/S130A/S162A | 7 |
| S20A/S25A/S99A/S100A/S101A/S102A/S130A/S162A/S79A/S81A | 8 |
| C29A       | 1                           |
| C64A       | 1                           |
| C134A      | 1                           |
| C141A      | 1                           |
| C29A/C141A | 2                           |
| C134A/C141A | 2                           |
A second set of stepwise deletions starting from the C terminus of BUS was tested in the background of BUS-DBD and NT-B (Fig. 3B). Interestingly, deletion of the C-terminal 30 amino acids (136–165) reduced transcription by more than 90\% in both backgrounds. All residual activity was lost with further C-terminal truncations. We conclude that a second region at the C terminus of BUS is also important for AF3 activity.

Constructs containing deletions from both the N- and C-terminal ends of BUS are shown in Fig. 3C. These double deletions generally support the conclusions drawn from the single deletions above, namely that AF3 activity depends on two BUS regions. Deletions up to amino acid 54 at the N terminus and back to amino acid 154 at the C terminus had minimal effects. (Note that the effects in BUS-DBD are more severe than they are in NT-B or PR-B.) These data suggest that the core region between amino acids 54 and 154 is sufficient for AF3 activity. Additional encroachment into this region from either side markedly reduces AF3 activity, particularly in the context of BUS-DBD. The residual construct containing amino acids 84–120 lacks transcriptional activity in all three BUS backgrounds. Taken together the data suggest that BUS contains two regions critical for AF3 activity as follows: region 1 (R1) between amino acids 54–84 and region 2 (R2) between 120–154. BUS-DBD activity is severely impaired when either R1 or R2 is deleted individually. Deletion of either R1 or R2 individually also significantly impairs NT-B and PR-B, but they retain some activity, presumably due to the actions of the second R, together with AF1 and/or AF2. Thus, R1 or R2 appears to be capable of some independent activity but only in the larger constructs. However, the other AFs were unable to support this activity when R1 and R2 were deleted together. Table II quantifies these data and includes the statistical analyses.

**Fig. 2. Protein expression of PR-B N-terminal deletion constructs.** Expression vectors for full-length PR-B and N-terminal deletions in PR-B were transiently transfected into COS monkey kidney cells. Cells were collected 48 h post-transfection at which time whole cell lysates were prepared. Samples were normalized to a SV40-β-galactosidase internal control and separated on a 7.5\% SDS-PAGE gel. Proteins were transferred to nitrocellulose and probed with a PR-specific monoclonal antibody AB-52 (28). Bands were visualized by enhanced chemiluminescence. Lane 1, PR-B amino acids 1–933; lane 2, PR-B ∆23 amino acids 24–933; lane 3, PR-B ∆54 amino acids 55–933; lane 4, PR-B ∆84 amino acids 85–933; lane 5, PR-B ∆114 amino acids 115–933; lane 6, PR-B ∆135 amino acids 136–933; lane 7, PR-B ∆148 amino acids 149–933; lane 8, PR-A amino acids 165–933.

**Fig. 3. Transcriptional activity of PR BUS deletion constructs.** The transcriptional activities of 17 PR mutants with deletions in BUS were tested in three backgrounds: BUS-DBD, NT-B, and PR-B. All assays were carried out in HeLa cells using a PRE2-TATA-luciferase reporter. Results are depicted as relative luciferase activity (firefly/Renilla) with transcription mediated by wild-type BUS-DBD, NT-B, and PR-B set at 100\%. Three types of deletions were tested: A, deletions at the N terminus of BUS; B, deletions at the C terminus of BUS (BUS-DBD and NT-B only); and C, deletions made at both ends of BUS. The amino acids present in BUS in each construct are illustrated on the left of the graph. The vertical line represents 100\% activity by wild-type constructs in A–C. Instances in which deletions were not tested in PR-B are indicated in C (ND). Relative activities and standard deviations for deletion mutants in BUS-DBD, NT-B, and in full-length PR-B are summarized numerically in Table II.

**Fig. 4B.** Two LXXLL motifs are found at the beginning of R1 (amino acids 55–59) and at the N-terminal edge of R2 (amino acids 115–119). Several amino acids that lie in the hydrophilic peak of R1 are indicated: Pro-69, Ser-70, Gln-78, and Gln-79. A single tryptophan residue lies in the second hydrophobic peak of R2 (Trp-140). Additionally, proline residues in each of the two hydrophilic regions of R2 are indicated: Pro-131, Pro-132, and Pro-151, Pro-152.

Therefore, a series of site-directed mutants targeting these potentially important amino acids was generated in R1 and R2 and cloned into the background of BUS-DBD. Most of these mutants were also transferred to NT-B and PR-B. Several of these mutations had little effect on AF3 activity (Table III, part a). However, mutation of several key amino acids in BUS resulted in major decrements in AF3 activity (Table III, part b). Detailed dose-response studies for these mutants are shown in Fig. 5. Expression vectors for these mutants were transfected into HeLa cells at increasing concentrations, and transcription was measured using the PRE2-TATA-luciferase reporter. In R1, proline 69 and serine 70 were singly or doubly mutated to
The bars in column 1 graphically depict the amino acid region(s) of BUS that were present in the constructs tested. Column 2 refers to the specific amino acids present in BUS by number. Deletions were placed in the context of three PR constructs as follows: column 3, BUS-DBD (amino acids 1–165, 556–645); column 4, NT-B (amino acids 1–645); and column 5, full-length PR-B (amino acids 1–933). Instances where the deletion was not tested in full-length PR-B are labeled as not determined (ND). Transcriptional activity was measured from a PRE-TATA-luciferase promoter in HEla cells (see under Experimental Procedures) and is expressed as the percent of activity ± S.D. compared with wild-type (w.t.) BUS-DBD (500 ng), NT-B (500 ng), or PR-B (25 ng) (each set at 100%). The synthetic progestin R5020 (10 nM) was added to each PR-B sample for 24 h. All numbers represent at least four data points from at least two separate experiments. * refers to transcription in the absence of BUS as follows: column 3, DBD alone (amino acids 556–645); column 4, NT-A (amino acids 166–645); and column 5, PR-A (amino acids 166–933).

| BUS region | Amino acids | BUS-DBD | NT-B | PR-B |
|------------|-------------|---------|------|------|
| 1–165 w.t. | 100.0       | 100.0   |      |      |
| 2–165      | 135.9 ± 15.6| 166.5 ± 6.1| 179.5 ± 7.0|
| 64–646     | 74.0 ± 4.3  | 110.7 ± 4.5| 101.4 ± 7.0|
| 84–165     | 15.7 ± 7.5  | 35.7 ± 8.1| 33.5 ± 6.6|
| 114–165    | 4.5 ± 2.3   | 27.4 ± 8.9| 15.7 ± 6.6|
| 135–165    | 2.0 ± 7.5   | 36.1 ± 4.5| 15.7 ± 1.4|
| 146–165    | 0.5 ± 3.2   | 18.4 ± 5.4| 6.3 ± 3.0|
| 1–135      | 7.5 ± 1.9   | 7.3 ± 0.6| ND   |
| 1–156      | 4.2 ± 0.8   | 3.7 ± 1.5| ND   |
| 1–75       | 8.0 ± 1.1   | 12.1 ± 3.0| ND   |
| 1–45       | 5.5 ± 0.8   | 2.8 ± 0.6| ND   |
| 54–154     | 72.0 ± 3.5  | 115.9 ± 10.5| 97.3 ± 11.2|
| 54–160     | 21.2 ± 2.4  | 44.9 ± 6.7| ND   |
| 54–130     | 0.4 ± 0.4   | 28.0 ± 4.9| ND   |
| 54–120     | 0.2 ± 0.5   | 36.6 ± 12.8| 26.4 ± 3.5|
| 84–154     | 8.3 ± 1.7   | 50.3 ± 8.0| 26.9 ± 9.2|
| 84–120     | 0.5 ± 0.4   | 7.4 ± 1.8| 1.8 ± 1.2|
| 1–54, 64–165| 26.8 ± 0.2| 44.8 ± 10.6| 44 ± 9.0|
| None*      | 0.0 ± 0.0   | 7.9 ± 3.1| 0.0 ± 2.9|

A Glycine and alanine, respectively. The single mutants only weakly (20–25%) impaired activity, but the double mutant resulted in a 60% decrease in BUS-DBD activity (Fig. 5c). The double mutant was also cloned into the NT-B and PR-B backgrounds, and the resultant receptors also had a 60% decrement in activity (Fig. 5, b and c). In full-length PR-B, the presence of the HBD led to transcriptional squelching with increasing receptor concentrations as described in Fig. 1. In R2, tryptophan 140 was targeted for mutation. It lies in one of the two hydrophobic peaks in this region. In AFs of other nuclear receptors, glucocorticoid receptor (GR) r1 and vitamin D receptor AF2, for example, tryptophan residues have been shown to be important for activation (37, 38). Remarkably, the single point mutation of W140A was sufficient to reduce AF3 activity by more than 70%. When L1 and L2 were mutated together, BUS-DBD activity was reduced by more than 85%. The L1 and L2 leucine mutants were also cloned singly or doubly into the backgrounds of NT-B and PR-B. These mutations produced major decrements in function, reducing transcriptional activity of these two receptor forms by 80–95%. In general, mutation of L2 generated a somewhat more severe phenotype than mutation of L1. Clearly, these L boxes play a key role in the function of AF3. BUS Loss-of-Function Mutations Are Promoter- and Cell Type-independent—The PRE<sub>2</sub>-TATA promoter is synthetic and simple. The progesterin-responsive MMTV promoter, in contrast, is natural and complex and includes a palindromic PRE plus three PRE half-sites. We therefore asked whether any of the two interesting mutants tested with the PRE<sub>2</sub>-TATA promoter above would have similar phenotypes on the MMTV promoter. To that end, the W140A and L1 mutants, cloned into the background of full-length PR-B, were compared with wild-type PR-B and PR-A. HeLa cells were transfected with 5–500 ng of the receptor expression vectors and 2 μg of the MMTV-luciferase reporter and were treated with R5020 (Fig. 6). Data were normalized to the Renilla luciferase control. Clearly, even on the MMTV promoter, mutation of three leucine residues in L1 or mutation of the single tryptophan residue in W140A severely impaired the activity of PR-B to levels more closely resembling the activity of PR-A. Therefore, even on the more complex promoter, mutations in L1 or R2 have a profound effect on the activity of PR-B.

To assess the cell type specificity of the effects, several mutants were transfected into PB-negative T47D breast cancer cells (30). Fig. 7 shows the resultant luciferase activity of PR-B, PR-A, and several BUS mutants in the context of PR-B on the
Column 1 refers to the specific amino acid mutations that were placed in the following three constructs: BUS-DBD, NT-B, and full-length PR-B. Transcriptional activity was measured in HeLa cells from a PRE$_2$-TATA-luciferase promoter. Results were normalized (SV40-PRE$_2$-TATA reporter. Wild-type PR-A have $\pm$ S.D. Instances where mutations were not assayed are labeled as not determined (ND). The synthetic progestin R5020 (10 nM) was added to PR-B samples for 24 h. All numbers represent a minimum of four data points from at least two separate experiments.

| Amino acid substitutions that reduce transcription by less than 25% | BUS-DBD | NT-B | PR-B |
|---|---|---|---|
| Wild type | 100.0 | 100.0 | 100.0 |
| P69G | 75.2 ± 0.5 | ND | ND |
| S70A | 83.1 ± 1.8 | ND | ND |
| Q77A/Q78A | 104.3 ± 26.5 | 85.7 ± 12.1 | 105.1 ± 13.1 |
| P131A/P132A | 101.0 ± 4.0 | ND | ND |
| P151A/P152A | 79.0 ± 3.8 | ND | ND |
| Amino acid substitutions that reduce transcription by more than 50% | P69G/S70A | L55A/L58A/L59A | L115A/L117A/L118A |
| | 44.0 ± 1.0 | 43.6 ± 4.3 | 41.6 ± 9.3 |
| | 26.9 ± 3.3 | 39.5 ± 8.4 | 19.5 ± 26.9 |
| | 26.3 ± 2.0 | 26.7 ± 1.3 | 20.7 ± 1.3 |
| | 12.7 ± 3.9 | 15.3 ± 1.7 | 4.7 ± 0.8 |
| W140R | 22.7 ± 3.7 | 23.1 ± 5.1 | 37.0 ± 13.8 |
| W140F | 47.6 ± 0.3 | 40.0 ± 4.4 | 43.7 ± 8.0 |
| W140A | 21.6 ± 5.9 | 22.5 ± 4.9 | 22.3 ± 4.9 |

**DISCUSSION**

**BUS, Autonomous or Modulatory AF?**—Previous studies (6, 8) demonstrate that BUS (residues 1–164 of PR-B) can strongly and autonomously activate transcription when linked to the homologous PR DBD. However, it is only weakly active when linked to the Gal4 DBD and completely inactive when linked to the estrogen receptor DBD (data not shown). These characteristics define BUS as an activation domain that functions autonomously in a “context-dependent” manner. Similar activation domains have been identified in USC2, ATF2, SRF, and Oct4 transcription factors (for review see Ref. 41). Additionally, BUS appears to modulate, perhaps even synergize with, the activities of the other PR AFs. For example, mutations of L1 and L2 (Fig. 5g) or the conserved tryptophan 140 (Fig. 5d), which all cripple AF3 activity (in BUS-DBD), have identical effects when the mutant AF3 is combined with AF1 in NT-B (Fig. 5, e and h) or with AF1 and AF2 in PR-B (Fig. 5, i and f). Thus even in the context of full-length B-receptors, the other AFs exhibit little independent activity in the absence of a functional AF3. This may explain why PR-A are usually weak receptors (6, 9).

Mechanisms of BUS Action: Intramolecular Contacts, Co-regulator Binding, Long Range Allostery?—Several mechanisms can be suggested to explain the strong transcriptional activity of BUS. 1) BUS may act by binding intramolecularly (or intermolecularly in the dimer) to the HBD. We have identified two NR box motifs (L1 and L2) in BUS that drastically reduce PR-B activity when they are mutated. The downstream L2 motif is the only region in BUS consistently predicted to form a helical structure with amphipathic properties. If this site is involved in protein-protein interactions, to what does it bind? One possibility is that it interacts with the co-activator-binding site in the HBD. There is evidence for N-C-terminal interactions both in PR and other steroid receptors. In vitro pull-down and in vivo mammalian two-hybrid interaction assays suggest that sequences within the N- and C-terminal regions of PR, androgen receptor and estrogen receptor, communicate in a ligand-dependent manner (42–45). Whether this interaction is direct and/or mediated by a co-regulator protein with binding sites in both regions is unclear. However, several candidate co-regulators, including ADA2, CREB, TBP, MMS19, TSG101, DRIP150, and SRC1, have recently been identified (46–50). Interestingly, studies with androgen receptor demonstrate that N/C-terminal interactions are mediated by motifs (FXXL and WXFL) in the N terminus bearing similarity to the signature LXXLL NR box motif (51). The authors postulate that an amphipathic helix formed by this motif binds to the hydrophobic pocket of the HBD. This pocket can also be occupied by NR box-containing, p160 co-activators, suggesting an important modulatory role for the N terminus in controlling co-activator binding at the HBD.

2) However, N/C-terminal interactions cannot be the sole mechanism for BUS function, since AF3 activity is maintained even when the HBD is deleted (see NT-B, Fig. 1c). In this regard, BUS also exhibits properties indicating that it is itself the target for protein binding. When BUS is linked to the Gal4 DBD, it squelches the activity of PR-B and PR-A, suggesting that BUS binds an activator protein present in limiting amounts (8).

3) That BUS function is context-dependent may be an important clue to its mechanism of action. The specific functional requirement of BUS for its homologous DBD suggests that an allosteric mechanism may be operative in PR. In this regard, studies with GR demonstrate that N-terminal regions can be allosterically modified by the promoter recognition sequence, and this influences the transcriptional phenotype (52). Recent NMR analyses show that single point mutations in the GR DBD mimic the DNA-bound or transcriptionally active conformation of the DBD (53). This provides structural evidence for long range allosteric effects from promoter recognition sequences, through the DBD, to the N-terminal AF (1) of GR. This communication is mediated in part by a transition in the packing arrangement of the DBD hydrophobic core.

4) Finally, it is possible that the intramolecular contact site of BUS is with its own DBD. This is not entirely unprecedented. Direct intramolecular contact between its DBD and an N-terminal activation region has recently been demonstrated by circular dichroism and proteolytic analyses of the Ets-1 transcription factor (54).

**Functional Map of BUS**—Systematic N- and C-terminal deletions of BUS cloned upstream of the PR DBD reveal a 101-residue “core” AF3 domain (residues 54–154; Fig. 3) analogous...
to one found in GR r1 (55). This minimal domain retains near wild-type activity in the BUS-DBD, NT-B, and full-length PR-B backgrounds. However, additional deletions from either end result in severe losses of activity, particularly in BUS-DBD.

The core fragment contains two serine residues (Ser-81 and Ser-102) that are phosphorylated in vivo (32, 35, 56). Their mutation had no effect on PR transcriptional activity either in

FIG. 5. Mutation of critical amino acids in R1 and R2 reduce BUS-mediated transcription. PR constructs containing point mutants in BUS were transiently transfected into HeLa cells and tested on a PRE2-TATA-luciferase reporter. Mutants were tested in three expression vector backgrounds at a range of concentrations from 10 to 500 ng: BUS-DBD (a, d, and g), NT-B (b, e, and h), and PR-B (c, f, and i). For PR-B samples 10 nM of the synthetic progestin R5020 was added for 24 h. Mutations tested include: P69A, S70A, and P69A/S70A (a–c), W140F, W140R, and W140A (d–f); L55A/L58A/L59A (L1), and L115A/L118A/L119A (L2) (g–i). Results are plotted as relative luciferase activity (firefly/Renilla) with the highest activity in each group set at 100%. Relative activities and standard deviations for BUS point mutants are summarized in Table III.

FIG. 6. Critical AF3 mutants affect PR transcription similarly from the MMTV promoter. Wild-type PR-B, PR-A, and PR-B containing mutants W140A and L1 (L55A/L58A/L59A) were transiently transfected into HeLa cells together with a MMTV-luciferase reporter construct at a range of expression vector concentrations from 5 to 500 ng. R5020 was added at concentrations of 10 nM for 24 h prior to harvest. Results are shown as relative luciferase activity (firefly/Renilla) with the highest PR-B point set at 100%. Data represent the average of duplicates from one representative experiment.

FIG. 7. AF3 mutants affect PR transcription similarly in T47D human breast cancer cells. Empty vector, wild-type PR-B (B), PR-A (A), and PR-B containing mutants (P69G/S70A), W140A, L1 (L55A/L58A/L59A), L2 (L115A/L118A/L119A), or (Q77A/Q78A) were transiently transfected into PR-negative T47D breast cancer cells (30). R5020 was added at concentrations of 10 nM for 24 h prior to harvest. Results are shown as relative luciferase activity (firefly/Renilla) with the highest PR-B point set at 100%. Data represent triplicate data points with standard deviations shown.

result in severe losses of activity, particularly in BUS-DBD. The core fragment contains two serine residues (Ser-81 and Ser-102) that are phosphorylated in vivo (32, 35, 56). Their mutation had no effect on PR transcriptional activity either in
trifluoroethanol (24). Attempts to crystallize these unstructured AFs have thus far been unsuccessful. Also, like GR r1, a smaller core domain of BUS, rich in hydrophobic residues, is critical for activity even though it is acidic in composition and highly phosphorylated. Mutagenesis surveys of VP16 (64), NF-xB (60), RARg2 (58), GCN4 (65), and GR r1 AFs, some of which are also acidic in character, clearly demonstrate that hydrophobic residues are critical for transcriptional activity. This is the case even when these residues are present in diverse contexts such as the well defined amphipathic helix of the CREB KID domain (66), the putative consensus activation surface motifs of RARg2 (Ψ-x-Ψ-Ψ), VP16, Rta, and GR r1 (58), and the structurally undefined and positionally flexible hydrophobic clusters of GCN4 (67). In fact, the L2 subdomain of BUS, which is critical for AF3 activity (Fig. 5), is the only region predicted to form an amphipathic α-helical structure (68). Sequence similarities among leucine-rich activation regions, NR boxes, and AP subdomains, exhibiting helical potential or shared “consensus” motifs, are consistent with the hypothesis proposed by Almlof et al. (69) that these regions provide a “common structural scaffold” required for interactions with co-regulatory proteins or for intramolecular contacts. The fact that critical mutations in two leucine-rich regions and the tryptophan residue elicited similar effects on all B-receptor backgrounds in a promoter-independent manner underscores the probability that these mutations alter a process that links BUS structure to the function of full-length PR-B in a fundamental way.

Acknowledgments—We are grateful to D. Toft (Mayo Clinic) for the gift of the polyclonal anti-PR-DBD antibody. We also thank S. Nordeen (University of Colorado Health Sciences Center) for providing us with the MMTV-luciferase reporter.

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D. L. Bain and K. B. Horwitz, unpublished data.

BUS-DBD or PR-B (34). This too is similar to GR r1, where mutation of five serine residues known to be phosphorylated in vivo had no effect on r1-related activity (57). At present it is not known whether Ser-70 is among the multiple phosphorylation sites in BUS although the combined P69G/S70A double mutant did reduce transcription by nearly 60%, raising the possibility that Ser-70 is phosphorylated and Pro-69 is necessary to fix it in a favorable surface-exposed orientation. There are also four cysteine residues in BUS capable of forming disulfide bonds, but their mutation, either individually or in pairs, had no detectable effect on activity. Thus, neither phosphorylation nor disulfide bond formation appear to play a fundamental role in the autonomous or modulatory transcriptional properties of BUS. The mutations, P69G, S70A, and Q77A/Q78A, within the single, most prominent hydrophilic peak of BUS (see Fig. 4) were also uninformative. Although Gln-75, Gln-77, and Gln-78 form a short glutamine-rich stretch, they do not appear to form a surface-exposed interaction site with a specific residue requirement.

Within the core domain, deletion of two regions, R1 (residues 54–84) and R2 (residues 120–154), produced the largest decre- ment in BUS-DBD, NT-B, or PR-B activity. A prominent hydrophobic peak in R1 contains a leucine-rich sequence (L1, residues 55–59) identical to the LXXNL NR box motif required for co-activator binding to nuclear receptor HBDs. Mutation of these leucines to alanine led to profound losses of BUS-DBD, NT-B, or PR-B activity. Similarly, mutation of a second LXXLL motif (L2, residues 115–119), associated with a second major hydrophobic peak, caused similar activity losses in all three backgrounds. L1 and L2 also bear a striking resemblance to a consensus motif predicted to form an amphipatic helix (Ψ-x-Ψ-Ψ) and identified in activation domains from several trans- activator proteins including GR r1, RARg2, VP16, and Rta, where Ψ is a hydrophobic/aromatic residue, where x is any residue, and – is an acidic residue (58). L1 and L2 match 5/6 and 4/6 positions of this consensus, respectively.

A third major hydrophobic peak in R2 surrounds tryptophan 140. Its single-point mutation to arginine (W140R) or alanine (W140A) reduced transcription of all three B-receptor backgrounds by more than 80%. Even mutation to phenylalanine, which conserves hydrophobicity at this position, led to a 50–60% loss of activity. Mutation of an analogous tryptophan in GR r1 (residue 234), to phenylalanine, had no effect (37), whereas mutation to glutamic acid reduced activity by more than 50%. Thus, hydrophobicity may not be the primary determin- ant of transcriptional phenotype. Rather than forming part of a loosely defined hydrophobic cluster or patch, the trypto-phan side chain may participate in a protein interaction having highly specific stereochemical requirements. The fact that W140A (uncharged) and W140R (charged) mutations resulted in identical activity losses also supports the notion that hydro-phobicity alone is not the primary specificity determinant at this position.

Relating Structural Properties to Function—The present study revealed striking parallels between AF3 of PR-B and the AFs of several related and unrelated transcription factors. The basis for these shared characteristics may lie in their analogous physicochemical properties. Like the AFs of VP16 (58), NF-xB (60), p53 (61), CREB (62), GR (63), and other transcription factors, AF3 is largely unstructured in aqueous solutions but has a propensity to form helices in hydrophobic solvents like trifluoroethanol (24). Attempts to crystallize these unstructured AFs have thus far been unsuccessful. Also, like GR r1, a smaller core domain of BUS, rich in hydrophobic residues, is...
