An N-terminal Inhibitory Function, IF, Suppresses Transcription by the A-isoform but Not the B-isoform of Human Progesterone Receptors*  

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The B-isoform of human progesterone receptors (PR) contains three activation functions (AF3, AF1, and AF2), two of which (AF1 and AF2) are shared with the A-isoform. AF3 is in the B-upstream segment (BUS), the far N-terminal 164 amino acids of B-receptors; AF1 is in the 392-amino acid N-terminal region common to both receptors; and AF2 is in the C-terminal hormone binding domain. B-receptors are usually stronger transactivators than A-receptors due to transcriptional synergism between AF1 and one of the two downstream AFs. We now show that the N terminus of PR common to both isoforms contains an inhibitory function (IF) located in a 292-amino acid segment lying upstream of AF1. IF represses the activity of A-receptors but is not inhibitory in the context of B-receptors due to constraints imparted by BUS. As a result, IF inhibits AF1 or AF2 but not AF3, regardless of the position of IF relative to BUS. IF is functionally independent and strongly represses transcription when it is fused upstream of estrogen receptors. These data demonstrate the existence of a novel, transferable inhibitory function, mapping to the PR N terminus, which begins to assign specific roles to this large undefined region.

Transcriptional control in response to extracellular signals involves the binding of regulatory proteins to specific enhancer elements of target genes. These proteins contain activation functions (AFs) through which contact is made with the basal transcription machinery either directly or indirectly by means of intermediary coregulatory proteins (1). Progesterone receptors (PR) are members of the nuclear receptor family of ligand-inducible transcription factors. These are structurally complex proteins containing multiple functional domains, including a highly conserved central DNA-binding domain (DBD), a moderately well conserved C-terminal hormone-binding domain (HBD), and a poorly conserved, N-terminal region whose function is largely unknown (1).

There are two naturally occurring isoforms of PR. The 933-amino acid B-receptors contain an N-terminal 164-amino acid upstream segment (BUS) that is missing in the truncated 769-amino acid A-receptors (2–5). The two PR isoforms have AF1 and AF2 in common (5, 6). AF1 maps to a 91-amino acid “proline-rich” segment located just upstream of the DBD and AF2 is located in the HBD (6). BUS, restricted to B-receptors, contains AF3 (5). In general, B-receptors are stronger transactivators than A-receptors (5, 7–9), and only B-receptors can activate transcription in the presence of antiprogestins (9–11). On the other hand, A-receptors can dominantly inhibit B-receptors (9, 12, 13) as well as other members of the steroid receptor family (14).

In addition to AFs, some transcription factors also contain inhibitory domains (IDs) that modulate the activity of the AFs. Such IDs have been identified by deletion mutagenesis that generate proteins with enhanced transcriptional activities. Examples include members of the AP1 family c-Jun (15), c-Fos, and the related protein, FosB (16); ATF-2, a member of the ATF/CAMP regulatory element-binding protein subfamily of basic region leucine zipper (bZIP)-containing transcription factors (17); and the lymphoid-specific transcription factor, Oct-2a (18). An ID has also been found in the proto-oncogene c-Myb, which plays a key role in hematopoiesis (19). Finally, IDs have been characterized in two yeast transcription factors: PHO4, which is regulated by phosphate levels (20), and ADR1, which regulates glycerol metabolism genes (21). To date, no ID has been described in the nuclear receptor family of transcription factors.

The IDs are structurally distinct from the AFs that they regulate (15–21). In some cases, inhibition is transferable to heterologous AFs, suggesting that the IDs are functionally independent. For example, when fused to the Escherichia coli polypeptide B42, the inhibitory regions of ADR1 repress transcription (21). Similarly, the IDs of c-Myb and c-Jun can inhibit the activity of VP16, a potent transactivator (15, 19). Inhibition by other IDs, however, is restricted to either the cognate AFs or a certain subset of AFs. For example, the bZIP domain of ATF-2 inhibits the related AFs of ATF-2 and E1a but not the acidic AF of VP16 or the glutamine-rich AF of Sp1 (17). Similarly, the N-terminal ID of c-Fos specifically silences the HOB1 subset of AFs found in c-Fos and c-Jun but not other phosphorylation-dependent AFs such as that found in cAMP regulatory element-binding protein (16).

Because A-receptors are weak transactivators compared
with B-receptors and are trans-dominant inhibitors of other nuclear receptors, we postulated that A-receptors contain inhibitory sequences distinct from the three defined AFs and that these sequences are inoperative in B-receptors. We sought this inhibitory function (IF) in a previously uncharacterized 292-amino acid region of the A-receptor N terminus. In this paper we have compared the activity of several PR constructs that either contain or lack IF. We show that IF expresses a novel inhibitory function, distinct from the AFs, that inhibits AF1 and AF2 but not AF3. Therefore, IF removal converts A-receptors from weak into strong transactivators. Additionally, IF is transferable and suppresses estrogen receptor (ER) activity when it is cloned upstream of ER.

**MATERIALS AND METHODS**

Recombinant Plasmids—Complementary DNAs, hPR2 and hPR1, encoding human A- and B-receptors, respectively, and HEGR, encoding human estrogen receptors, cloned into pSG5 (4), were gifts from P. Chambon (Strasbourg, France). Construction of BUS-BDB, N-terminal B (NTB)-DBD, N-terminal A (NTA)-DBD, DBD-HBD, and BUS-BDB-HBD expression vectors, all containing a nuclear localization signal (NLS), was described in Sartorius et al. (5).

IF was made by polymerase chain reaction (PCR) amplification of PR nucleotide (nt) sequences 2109–2678 encoding AF1, DBD, and the NLS (4). The 5′-sense primer contained an EcoRI site, a Kozak consensus sequence (22), and ATG initiation codon. The 3′-antisense primer contained a STOP codon and BglII site (5). The resulting PCR fragment was cloned into pSG5 digested with EcoRI and BglII, and the NLS (4). The 5′-sense primer contained an EcoRI site, a Kozak consensus sequence (22), and ATG initiation codon. The 3′-antisense primer contained a STOP codon and BglII site (5). The resulting PCR fragment was cloned into pSG5 digested with EcoRI/BglI. A-ΔIF was made by digesting NTγ-ΔIF with BssUI/BglII and isolating the larger fragment, which contains pSG5 sequences, AF1, and the DBD N terminus upstream of the BssI site. This was used as a recipient for ligation of the BssI/BglII fragment from BUS-BDB-HBD, which contains the DBD C terminus, NLS, and HBD. For B-ΔAF1 a fragment encoding sequences upstream of AF1 (fragment 1) was made by amplification of hPR1 sequences using a 5′-sense primer (nt 1855–1878) containing an MluI site and a 3′-antisense primer (nt 2096–2116) containing a SalI site. Fragment 2, which spans sequences from the 3′ border of AF1 to the end of the HBD, was made with a 5′-sense primer (nt 2385–2404) containing a SalI site and a 3′-antisense primer (nt 3525–3545) containing a BglII site (5). The two fragments were combined with an MluI/BglII vector-containing fragment (fragment 3) derived from hPR1. All three fragments were ligated to provide B-ΔAF1. A-ΔAF1 was made from B-ΔAF1 by removing the B-ΔAF1 EcoRI/BamHII fragment, filling in overhanging ends, and ligating the blunt ends. For NTγ-ΔAF1 fragment 4, which spans sequences from the 3′ end of AF1 through the NLS, was amplified from NTγ-BDB using the 5′-sense primer for fragment 2 containing a SalI site and a 3′-antisense primer (nt 2646–2678) containing a BglII site, coding sequences homologous to the 3′ end of NTγ-BDB and a STOP codon (5). PCR fragments 1 and 4 were combined with the MluI/BglII vector-containing fragment 3 and ligated.

IF-BUS-BDB was made by amplification of IF (spanning nt 1239–2108) from hPR1 using primer pairs containing EcoRI sites. BUS-BDB-NLS was amplified from BUS-BDB with a 5′-sense primer containing an EcoRI site and a 3′-antisense primer containing a BglII site and STOP codon (5). The two fragments were ligated and digested with MluI/BglII. The larger fragment was cloned into the vector-containing fragment derived from NTγ-BDB digested with MluI/BglII. IF-NLS was made by amplification of IF using a 5′-sense primer containing a BamHI site and a 3′-antisense primer containing an EcoRI site. This fragment was cloned into pSG5–2TK digested with EcoRI and BamHI. pSG5–2TK was made by digesting pSG5 with EcoRI and BglII and inserting an oligo linker containing a Kozak consensus sequence (22), BglII site, the PR NLS, multi-cloning sites for EcoRI, HI, and three STOP codons. IF-ER was made by amplification of ER using a 5′-sense primer containing an EcoRI site, a Kozak consensus sequence (22), and ATG initiation codon. The resulting fragment was cloned into pSG5 digested with EcoRI/BglII. IF was by polymerase chain reaction (PCR) amplification of PR nucleotide (nt) sequences 2109–2678 encoding AF1, DBD, and the NLS (4). The 5′-sense primer contained an EcoRI site, a Kozak consensus sequence (22), and ATG initiation codon. The 3′-antisense primer contained a STOP codon and BglII site (5). The resulting PCR fragment was cloned into pSG5 digested with EcoRI/BglII. A-ΔIF was made by digesting NTγ-ΔIF with BssUI/BglII and isolating the larger fragment, which contains pSG5 sequences, AF1, and the DBD N terminus upstream of the BssI site. This was used as a recipient for ligation of the BssI/BglII fragment from BUS-BDB-HBD, which contains the DBD C terminus, NLS, and HBD.

**RESULTS**

A series of expression vectors was constructed in which each region of PR that contains an AF was fused, either alone or in combination with another AF, to the PR DBD-NLS (5). Additionally, the constructs contained or lacked IF, the 291 amino acids lying upstream of AF1. IF was also cloned upstream of full-length ER. The detailed structure of all the constructs is shown in Fig. 1.

Expression of the receptor proteins was verified by SDS-polyacrylamide gel electrophoresis and immunoblotting of whole-cell extracts derived from COS cells (Fig. 2). They range in size from 191 to 933 amino acids and are all well expressed. The presence of multiple bands for some constructs, particularly ones that contain BUS (Fig. 2, lane 8, for example), is due to phosphorylation (5, 26). Interestingly, this multiple banding pattern is amplified by removal of the HBD (Fig. 2A, lanes 4 and 8) and is reduced by juxtaposition of IF upstream of BUS (Fig. 2, compare lane 6 in A and lane 1 in B). Each of the receptors shown in Fig. 2 binds to a perfect palindromic PRE in an electrophoretic mobility shift assay (data not shown).

An Inhibitory Function—To search for an inhibitory function, an A-receptor variant was constructed (A-ΔIF) that lacks the N-terminal 292 amino acids located upstream of AF1 (Fig. 1). This previously uncharacterized domain, designated IF (amino acids 165–455), although common to both receptor isoforms, has the potential to function differently when free at the N terminus of A-receptors but constrained by BUS in B-receptors. Strikingly, on the PRE2-TATAtk promoter (Fig. 3A) or on the MMTV promoter (Fig. 3B), deletion of IF converts A-receptors to weak fragments to strong transactivators equivalent to B-receptors. However, unlike B-receptors, but like A-receptors, A-ΔIF displays strong “self-squelching” behavior. Therefore, as the concentration of A-ΔIF is increased, the high levels of CAT activity fall.

**IF Inhibits AF1 and AF2 but Not AF3**—Fig. 3 shows that IF contains a potent inhibitory function that suppresses the activity of A-receptors. However, because B-receptors, which also contain IF sequences, are strong transactivators, we postulated that IF does not influence AF3 but that its inhibitory effects are...
restricted to AF1 and/or AF2 (Fig. 4). To test this hypothesis, constructs were made that contained each AF alone, with or without IF (Fig. 1). Dose-response data using PRE2-TATAtk-CAT are shown in Fig. 4 for AF1 (NTA-DBD) with (1) and without (2) IF (Fig. 4A); AF2 (DBD-HBD) with (1) and without (2) IF (Fig. 4B); and AF3 (BUS-DBD) with (1) and without (2) IF (Fig. 4C) compared with full-length B-receptors. Analogous to its role in full-length A-receptors, we find that IF has its strongest effect on AF1 and AF2 at low receptor concentrations. Deletion of IF strongly increases AF1-dependent transcription (Fig. 4A, 100 ng) but its inhibitory effects on AF1 can be overcome at higher concentrations because of the failure of NTA-DBD to self-squelch. Note that NTA-DBD is the only A-receptor construct that does not self-squelch (not shown). These and other data (Fig. 4B and not shown) suggest that the HBD is required for this property.

Deletion of IF from an AF2-containing HBD construct also converts a weak transactivator into a stronger one at low concentrations (Fig. 4B). At higher concentrations, however, the influence of self-squelching abolishes AF2 activity. Thus,

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CAT are shown in Fig. 4 for AF1 (NTA-DBD) with (+) and without (−) IF (Fig. 4A); AF2 (DBD-HBD) with (+) and without (−) IF (Fig. 4B); and AF3 (BUS-DBD) with (+) and without (−) IF (Fig. 4C) compared with full-length B-receptors. Analogous to its role in full-length A-receptors, we find that IF has its strongest effect on AF1 and AF2 at low receptor concentrations.

Deletion of IF strongly increases AF1-dependent transcription (Fig. 4A, 100 ng) but its inhibitory effects on AF1 can be overcome at higher concentrations because of the failure of NTA-DBD to self-squelch. Note that NTA-DBD is the only A-receptor construct that does not self-squelch (not shown). These and other data (Fig. 4B and not shown) suggest that the HBD is required for this property.

Deletion of IF from an AF2-containing HBD construct also converts a weak transactivator into a stronger one at low concentrations (Fig. 4B). At higher concentrations, however, the influence of self-squelching abolishes AF2 activity. Thus,
the HBD mediates two opposing activities: a stimulatory AF2 function and an inhibitory self-squelching function. IF can suppress the former but has no effect on the latter. We speculate that IF inhibition of AF1 and AF2 is additive in full-length A-receptors, accounting for their weak activity at all concentrations.

Neither the inhibitory function of IF nor the self-squelching activity of the HBD operates in B-receptors. Removal of IF does not enhance AF3 activity at low concentrations (Fig. 4C) nor does it affect AF3 at higher concentrations, and, like full-length B-receptors (Fig. 3), the B-receptor derivatives BUS-DBD-HBD or B-ΔAF1 do not self-squelch despite presence of the HBD (data not shown).

**Mechanisms of PR Auto-inhibition by IF**—Several possible mechanisms can be invoked for auto-inhibition of A-receptors by IF. One is binding of a repressor at IF. However, we find that overexpression of an IF-NLS construct does not squelch the putative repressor (not shown). Another possible mechanism is steric hindrance of AF1 and AF2 by IF due to the latter’s putative repressor (not shown). However, we find that IF does not act simply by steric hindrance of AF1 and AF2 by IF due to the latter’s putative repressor (not shown). Another possible mechanism is steric hindrance of AF1 and AF2 by IF due to the latter’s putative repressor (not shown). However, we find that IF does not act simply by steric hindrance of AF1 and AF2 by IF due to the latter’s putative repressor (not shown). Another possible mechanism is steric hindrance of AF1 and AF2 by IF due to the latter’s putative repressor (not shown). However, we find that IF does not act simply by steric hindrance of AF1 and AF2 by IF due to the latter’s putative repressor (not shown). Another possible mechanism is steric hindrance of AF1 and AF2 by IF due to the latter’s putative repressor (not shown).

**IF Is Transferable to the Heterologous AFs of ER**—Inhibitory domains, like activation domains, can be discrete and modular. To determine whether IF effects were transferable, we tested the ability of IF to inhibit the heterologous AFs of ER. ERs contain AF1 and AF2 and, in this respect, structurally resemble A-receptors (14, 27, 28). However, ER have no sequences homologous to IF. To test the effects of IF on ER, an IF-ER chimera was constructed (Fig. 1) in which IF was cloned upstream of ER. Fig. 5A shows transcription by wild-type ER or IF-ER of the ERE<sub>α</sub>-TATA<sub>α</sub>-CAT reporter in the absence (open symbols) or presence of 10 nM 17β-estradiol (solid symbols). CAT activity induced by ER is maximal at 0.1 μg of the expression vector and then decreases at higher concentrations due to self-squelching. This has previously been described (7, 14). At the same cDNA concentrations, IF cloned upstream of ER markedly reduces transcription. Fig. 5B (B and C) compares the transcriptional efficacy of ER and IF-ER when the two are expressed at similar protein levels. We find that IF-ER is expressed at lower efficiency than ER. Thus, 1 μg of the IF-ER expression vector and 0.1 μg of HEGO produce equivalent amounts of immunoreactive protein (Fig. 5B). Note that the expected molecular mass of ER is 65 kDa and that of IF-ER is 97 kDa. Fig. 5C shows that at these equivalent protein concentrations, wild-type ER strongly activate transcription, whereas little or no transcription is produced by IF-ER. We conclude that when IF is transferred upstream of ER, it silences ER-dependent transcription.

**DISCUSSION**

This paper describes a novel, transferable inhibitory function, designated IF, which lies in the 292-amino acid N-terminal region upstream of the PR AF1 but operates only in the context of A-receptors.

**A- versus B-receptors**—Why progesterone target tissues contain two receptor isoforms remains an intriguing physiological puzzle. They were first described in chick oviducts (29) and then in human cells (2). In humans, the two proteins are the products of a single gene that has two promoters, from which at least nine messages, two of which are A-receptor specific, are transcribed (30). An internal AUG present in some messages may also encode A-receptors (30). Thus, there is complex regulatory control over protein levels of the two isoforms, the details of which are still unclear. In initial studies using breast cancer cell lines, the two isoforms were found in approximately equimolar amounts (3). However, it is now clear that their relative levels are under tight developmental and hormonal control in chicken oviducts (31–33) and the female rat brain (34), and preliminary data in the human uterus also show a discordance, with A:B ratios ranging between 50:1 and 2:1 during the menstrual cycle due to large excursions in the levels of B-receptors (35). In breast cancers 25% of tumors have a
significan excess of A-receptors (36). Given the functional transcriptional differences between the two isoforms, their unequal distribution in tissues and tumors could be biologically important. For example, an excess of B-receptors in the uterus may mark those patients at greatest risk of developing tamoxif-induced endometrial cancers (37).

Transferable Inhibition of AF1 and AF2—Much of the work devoted to understanding regulation of transcription by steroid receptors has focused on AFS and their stimulatory actions. However, transcriptional inhibition may be equally important as a way of preventing or terminating activation. Studies that deal with inhibition have focused on composite DNA elements and invoke mechanisms in which receptor occupancy at one DNA site interferes with transcription by an activator at an adjoining site (38, 39). Heterodimerization of an activator by a DNA site interferes with transcription by an activator at an adjoining site (38, 39). Heterodimerization of an activator by a DNA site interferes with transcription by an activator at an adjoining site (38, 39).

We show that IF markedly suppresses the transcriptional activity of AF1 and AF2 of A-receptors (Fig. 4). The ability of IF to also strongly suppress AF1 plus AF2 of ER (Fig. 5) suggests that its inhibitory mechanisms involve general steroid receptor-related processes. It is tempting to speculate that IF prevents the binding of key AF1 or AF2 transcriptional coregulators that are shared by all steroid receptors (40). However the inability of soluble IF (i.e. IF-NLS) to squelch such activity suggests that IF acts structurally, perhaps through intramolecular contacts. Our data show that the inhibitory activity can be transferred to the cognate AFS of ER. In that respect, IF resembles the bZIP domain of ATF-2 and the N-terminal ID of c-Fos (16, 17). Whether IF can also suppress heterologous AFS remains to be determined.

IF Cannot Inhibit AF3—B-receptors also contain the IF element, but its repressor activity appears to be constrained by BUS, which is located further upstream. Therefore, IF specifically inhibits AF1 and AF2 of PR but not AF3 (Fig. 4). Furthermore, IF cannot inhibit AF3 regardless of its position relative to BUS (Fig. 4, C and D). We have previously demonstrated that AF3 transcriptional activity is unusual in that it is critically dependent on the presence of the PR DBD. In gel mobility shift studies, BUS-DBD binds to a PRE only if a bivalent monoclonal antibody is added, which appears to supply a dimerization function. The possibility exists that BUS and the DBD of PR are linked through intramolecular contacts so that the mechanisms of AF3 action may be quite different from those of AF1 and AF2.

There is now compelling evidence that alterations in the three-dimensional structure of steroid receptors modifies their transcriptional behavior. Most of that work comes from analyses of the HBD. For example, using protease accessibility as a probe for receptor structure, it has been shown that PR (41) and ER (42, 43) assume altered conformational states when the HBD is occupied by agonists or antagonists. More recently, crystallographic analyses of the HBDs of unliganded RXXR (44) and liganded retinoic acid receptor γ (45) and thyroid receptor α1 (46) have yielded information about the position of residues critical to the function of AF2. These studies also show that binding of ligand alters the conformation of the HBD, which, depending on the ligand, may be interpreted as a positive or negative signal by the transcriptional machinery (41).

The present studies focus on the PR N terminus to explain functional differences between the two isoforms. As yet, nothing is known about the three-dimensional structure of the N termini of any nuclear receptor. This region is, however, structurally the most divergent among members of this family of proteins, suggesting that each receptor will take on unique structural conformational changes that determine its specificity. We postulate that structural differences between the two isoforms, due to the presence or absence of BUS, influence the functional role of IF.

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