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# Nongenomic Activity of Ligands in the Association of Androgen Receptor with Src

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**ABSTRACT** Androgen receptor (AR) induces cell proliferation by increasing the kinase activity of Src. We describe an approach for discriminating agonist and antagonist in a nongenomic steroid-signaling pathway using an association of AR with Src. We constructed a pair of genetically encoded indicators, where N- and C-terminal fragments of split firefly luciferase (FLuc) were fused to AR and Src, respectively. The fusion proteins with AR and Src are localized in the cytoplasm and on the plasma membrane, respectively. Upon being activated with androgen, AR undergoes an intramolecular conformational change and binds with Src. The association causes the complementation of the split FLuc and recovery of FLuc activity. The resulting luminescence intensities were taken as a measure of the rapid hormonal activity of steroids in the nongenomic AR signaling. Ten minutes are required for the AR–Src association by 5α-dihydroxytestosterone (DHT), which was completely inhibited by an antagonist, cyproterone acetate. The activities of ligands in the nongenomic pathway of AR were compared with those in the genomic pathway obtained on the basis of the nuclear trafficking of AR in mammalian cells. The comparison revealed that DHT and testosterone activate both genomic and nongenomic pathways of AR. 17β-Estradiol and progesterone were found to be specific activators only for the genomic signaling pathway of AR. On the other hand, procymidone exhibited a specific activity only for the nongenomic signaling pathway of AR. The present approach is the first example addressing the agonistic and antagonistic activities of ligands in a nongenomic pathway of AR.

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Human sex hormones exert some of their effects through the action of their receptors on gene expression. The ligands regulate gene transcription by interaction with intracellular nuclear receptors (NRs), which act as ligand-dependent transcription factors. The cell signaling for protein transcription by the ligand–receptor complex has been called the genomic pathway. The phenomena accompany coactivator recruitment and nuclear trafficking of NRs. On the other hand, a number of other effects of sex hormones such as a receptor–kinase interaction are too rapid to be explained in terms of the relation to the direct gene expression. These actions are known as nongenomic actions of NRs and are typically mediated through their membrane-associated receptors in the cytosolic compartment. The actions are frequently associated with the activation of various protein-kinase cascades (Figure 1, panel a) (*1*–*4*). Studies on the potential activities of sex hormones and synthetic chemicals on genomic and nongenomic signaling pathways are of value, because they are deeply related with cell growth and expression of sex phenotypes (*3*, *5*). Up to now, the agonistic and antagonistic activities of ligands in nongenomic pathways have not been explored, although it is believed that ligands play a critical role for stimulating genomic and/or nongenomic signaling pathways of NR in a physiological circumstance.

A male sex hormone, androgen, binds with the androgen receptor (AR) in the cytosol of mammalian cells and induces cell proliferation by increasing the kinase activity of Src located adjacent to the cell membrane (*6*, *7*). Estrogen also proliferates cell growth *via* activating an interaction of the estrogen receptor (ER) with Src (*6*–*8*). Sex hormones such as estrogen, progesterone,

and androgen can stimulate the Src/p21ras/extracellular-signal-regulated kinase (ERK) pathway in breast and prostate cancer cells (*6*, *9*, *10*). Because the tyrosine kinase domain of Src is an important target for anticancer drugs, the contribution of AR and ER to the Src pathway has gained much interest in cancer studies (*8*–*10*).

The interactions of Src with AR and/or ER in the nongenomic pathway were previously evidenced with coimmunoprecipitation (*6*). However, coimmunoprecipitation experiments provide limited information on the molecular dynamics in living cells, because the result was obtained with a destructive method with a long assay procedure. The association of AR with Src in the vicinity of plasma membranes cannot be determined with reporter gene assays including yeast or mammalian two-hybrid methods. It is therefore a valuable approach to determine interactions of sex hormone receptors with such proteins as Src in the cytosol of living cells. This development is especially useful for early screening of carcinogens and developing anticancer drugs.

We describe herein an approach to detecting activities of ligands based on the AR–Src association using a pair of genetically encoded bioluminescent indicators.

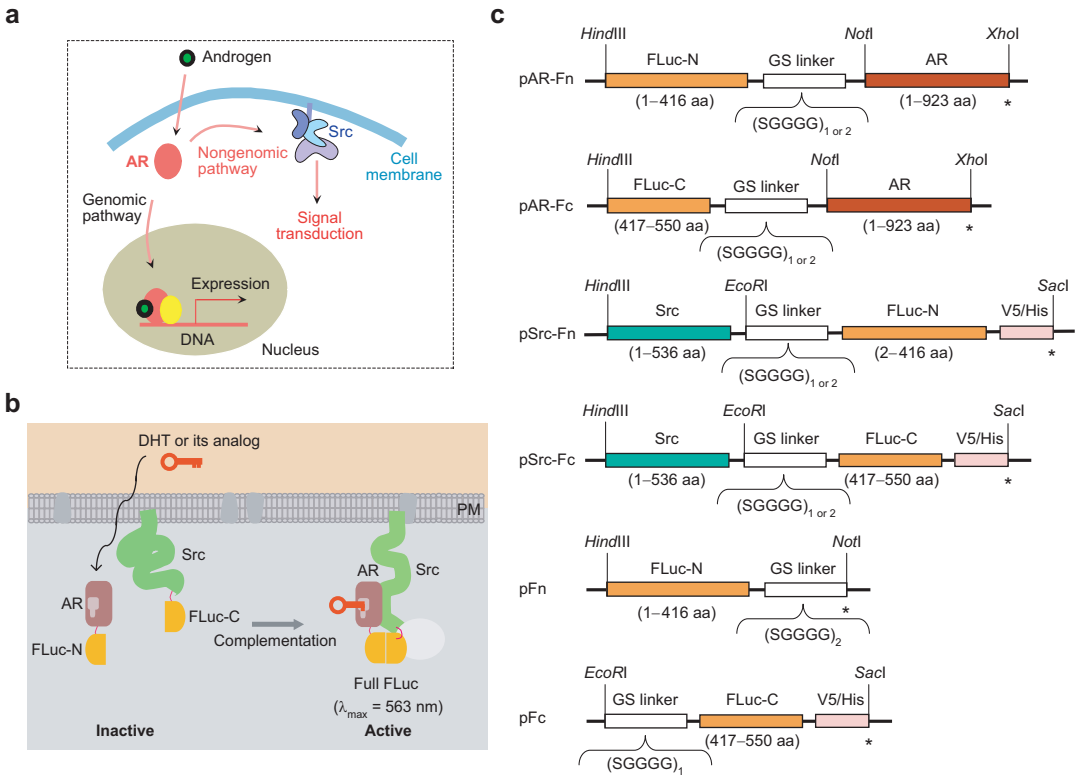


**Figure 1. A scheme for evaluating genomic and nongenomic activities of ligands based on protein interactions. a) Genomic and nongenomic pathways of AR signaling in response to androgen. b) Complementation of split FLuc upon androgen-induced AR–Src interaction. When MCF-7 cells carrying pAR-Fn10 and pSrc-Fc5 were stimulated with DHT or its analogs, the association between AR and Src was triggered. As a result of the association, the N- and C-terminal fragments of split FLuc are brought into proximity to complement. The luminescence intensities from the complemented FLuc were determined with a luminometer. c) Schematic structures of cDNA constructs. AR is attached to FLuc-N or FLuc-C through 5 or 10 GS linker peptides, i.e., SGGGG or SGGGGSGGGG. They were named pAR-Fn5, pAR-Fn10, pAR-Fc5, and pAR-Fc10, respectively. The numbers in the plasmid names, 5 and 10, refer to the linker lengths. Src is connected to FLuc-N or FLuc-C through the 5 or 10 GS linker peptides. They were named pSrc-Fn5, pSrc-Fn10, pSrc-Fc5, and pSrc-Fc10, respectively. V5/His indicates epitope sequences for antibody recognition. The specific amino acid sequence is** GKPIPNPLLGLDST**RTG**HH-HHHH**. The asterisks (*) represent stop codons.**

A basic concept for determining protein–protein interactions was previously proposed by us and other groups using a protein-fragment complementation assay with split firefly, *Gaussia*, or *Renilla* luciferase (*11*–*14*). The approach was a rational way to explore protein dynamics in the physiological context of living cells. A pair of genetically encoded indicators was constructed and co-expressed in human breast cancer-derived MCF-7 cells. One is composed of cDNAs of AR and N-terminal fragment of firefly luciferase (FLuc-N), designated as pAR-Fn, and the other with complementary DNA (cDNA) of Src and C-terminal fragment of firefly luciferase (FLuc-C), desig-

**TABLE 1. Eight kinds of plasmids constructed for an optimal AR–Src association. Each cDNA component consisting of the plasmids was specified in order.[a]**

| Plasmid number | Plasmid name | GS linker length | AR (1–920 aa) | Src (1–536 aa) | FLuc-N (1–416 aa) | FLuc-C (417–550 aa) |
|---|---|---|---|---|---|---|
| (1) | pAR-Fc5 | 5 | + | − | − | + |
| (2) | pAR-Fc10 | 10 | + | − | − | + |
| (3) | pAR-Fn5 | 5 | + | − | + | − |
| (4) | pAR-Fn10 | 10 | + | − | + | − |
| (5) | pSrc-Fc5 | 5 | − | + | − | + |
| (6) | pSrc-Fc10 | 10 | − | + | − | + |
| (7) | pSrc-Fn5 | 5 | − | + | + | − |
| (8) | pSrc-Fn10 | 10 | − | + | + | − |

[a]The symbols "+" and "−" designate the presence and absence, respectively, of the cDNA component.

nated as pSrc-Fc (Figure 1, panels b and c). The fusion proteins with AR and Src are localized on the cytoplasm and on the plasma membrane of MCF-7 cells, respectively. In the resting state without androgen, AR remains inactive. In the presence of androgen, AR binds with Src, and the N- and C-terminal fragments of FLuc come into proximity. Because of refolding of the FLuc from the complementary fragments (*15*), the luciferase activity is partially recovered. The luminescence intensities were estimated as a measure of the hormonal activity of the steroid in the nongenomic AR signaling. We have determined the hormonal effects of androgen agonists and antagonists on the AR–Src association as a typical nongenomic pathway of the steroid signaling. The results were compared with those based on a typical ligand-induced genomic pathway for the androgen signaling (*16, 17*). We will discuss the relative contribution of several ligands on the genomic and nongenomic signaling pathways.

## RESULTS AND DISCUSSION

**Construction of the Indicators and Evaluation of Their Sensitivity to Androgens.** Genetically encoded bioluminescent indicators were developed for determining the ligand-induced association of AR with Src, the ends of which were linked with each fragment of split FLuc (Figure 1, panels b and c). Here, FLuc was dissected between Gly416 and Trp417 for an efficient complementation of split FLuc, where the luminescence of the split FLuc was completely lost. We made eight kinds of new indicators for determining the best one for an efficient ligand-controlled recovery of luminescence intensities (Table 1). GS linker peptides consisting of 5 or 10 amino acids were inserted between AR and a fragment of FLuc, and between Src and the other fragment of FLuc, respectively, for ensuring flexibility of the domains and a maximum luminescence recovery. When AR is stimulated with androgen, AR interacts with Src. This interaction brings the FLuc-N and FLuc-C into proximity to initiate their complementation. The complemented FLuc allows quantitative evaluation of the hormonal activities of androgen or its analogue with a luminometer.

The sensitivity of each indicator to androgen was estimated in the presence or absence of $10^{-5}$ M 5$\alpha$-dihydroxytestosterone (DHT) (Figure 2, panel a). Among the constructed indicators, the MCF-7 cells with pAR-Fn10 and pSrc-Fc5 exhibited the best signal-to-background ratio. This is because they contain a suitable dissection point of FLuc (*18*) and optimal linker lengths for minimizing the steric hindrance and maximizing the ligand-induced recovery of FLuc activity.

We constructed two new probes without Src or AR for a negative control study as shown in Figure 1, panel c. The plasmids, pFn and pFc, respectively contain N- and C-terminal fragments of FLuc. MCF-7 cells were cotransfected with (i) pFn and pFc, (ii) pAR-Fn10 and pFc, (iii) pFn and pSrc-Fc5, or (iv) pSrc-Fc5 and pAR-Fn10. The respective luminescence intensities from the cells were estimated in the presence or absence of $10^{-5}$ M DHT (Figure 2, panel c). The results showed that cases (i), (ii), and (iii) exhibited no response to DHT, but only case (iv) provided a considerable increase of the luminescence intensities in response to DHT. It shows that the interaction of AR with Src in response to DHT is the only reason for the increase in the luminescence intensities.

It is known that binding of a ligand to the AR induces dissociation of heat shock protein 90 (Hsp90) from AR. However, the dissociation did not increase the nonspecific binding between the free N- and C-terminal fragments of FLuc. If the AR–Src binding was a passive interaction due to a simple increase of free AR, both cases (ii) and (iv) should have increased the luminescence intensities in response to DHT. However, that did not happen in the present control study.

**Evaluation of the Expressions of N- and C-Terminal Fusion Proteins with Western Blotting.** The expressions of the fusion proteins from pAR-Fn10 and pSrc-Fc5 in MCF-7 cells were determined with Western blotting (Figure 2, panel b).

The mouse anti-AR antibody recognized a specific band of 146 kDa, the size of which was the same as

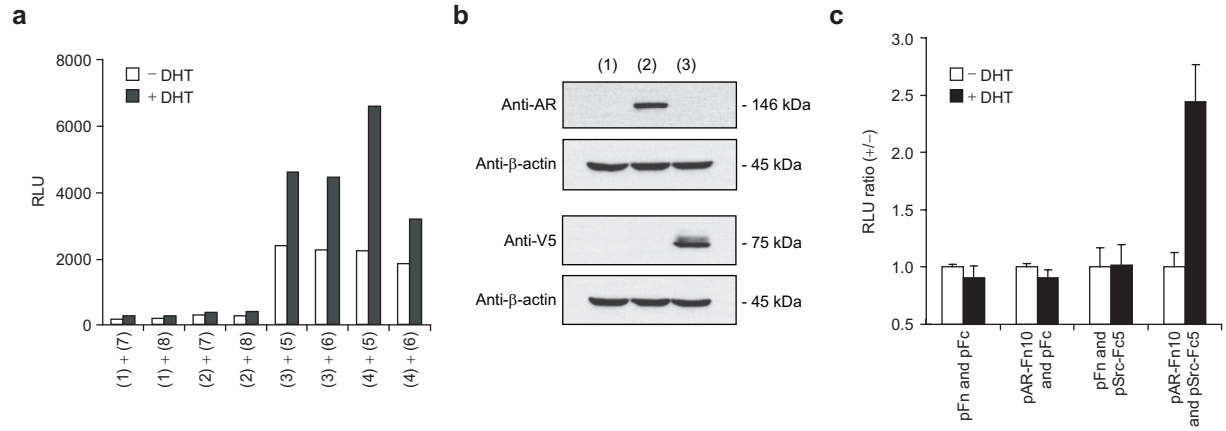


**Figure 2. Selection of optimal probes for protein interactions. a) Estimation of an optimal combination of the plasmids with different GS linkers for determining the DHT-activated AR–Src association. The MCF-7 cells carrying each pair of plasmids were stimulated with vehicle (0.1% (v/v) DMSO) (open bars) or $10^{-5}$ M DHT (solid bars) for 20 min. The resulting luminescence intensities were monitored. The numbers in the horizontal axis show the plasmid numbers specified in Table 1. b) Western blotting analysis of the expressions of pAR-Fn10 and pSrc-Fc5 in MCF-7 cells. Lanes 1–3 represent the lysate blots from intact MCF-7 cells, the cells carrying pAR-Fn10, and the cells carrying pSrc-Fc5, respectively. c) A negative control study for examining the contribution of nonspecific interactions between AR and Src in the present study. Two new plasmids, pFn and pFc, were made with N- or C-terminal fragments of FLuc alone. MCF-7 cells were cotransfected with (i) pFn and pFc, (ii) pAR-Fn10 and pFc, (iii) pFn and pSrc-Fc5, or (iv) pAR-Fn10 and pSrc-Fc5. The respective luminescence intensities from the cells were estimated in the presence or absence of $10^{-5}$ M DHT. The results show that only case (iv) provided a considerable increase in the luminescence intensities in response to DHT.**

that of the expected fusion protein containing AR and FLuc-N. In addition, the mouse anti-V5 epitope antibody recognized a specific band of 75 kDa. The protein size was identical with that of the fusion protein containing Src and FLuc-C. As a reference for the amounts of the electrophoresed proteins, a marker protein, β-actin, was stained with its specific antibody. The results with Western blotting show that (i) the fusion proteins are indeed expressed in MCF-7 cells, (ii) the sizes of the fusion proteins are the same as the expected ones, and

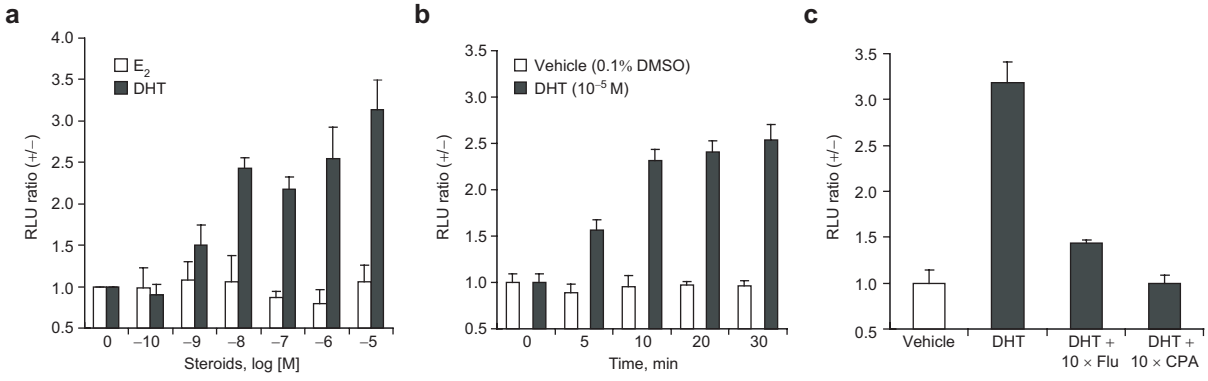


**Figure 3. Evaluation of probe performance in response to ligands. a) Dose–response graphs for DHT and $E_2$ based on the luminescence intensities from the recovered FLuc. The MCF-7 cells carrying pAR-Fn10 and pSrc-Fc5 were stimulated with differing concentrations of DHT (solid bars) or $E_2$ (open bars). Zero at the *x*-axis indicates a treatment with vehicle (0.1% (v/v) DMSO) ($n = 3$). b) Ligand-dependent time course of the luminescence intensities from MCF-7 cells carrying pAR-Fn10 and pSrc-Fc5. The cells were stimulated with vehicle (0.1% (v/v) DMSO) (open bars) or $10^{-5}$ M DHT (solid bars) for the times indicated in the horizontal axis. The luminescence intensities at each time were determined with a luminometer ($n = 3$). c) Inhibitory effects of flutamide and CPA on the bioluminescence intensities developed by $10^{-5}$ M DHT. MCF-7 cells carrying pAR-Fn10 and pSrc-Fc5 were stimulated with a $10^{-4}$ M concentration of flutamide or CPA. The cells were then additionally stimulated with $10^{-5}$ M DHT. The resultant luminescence intensities were recorded with a luminometer ($n = 3$).**

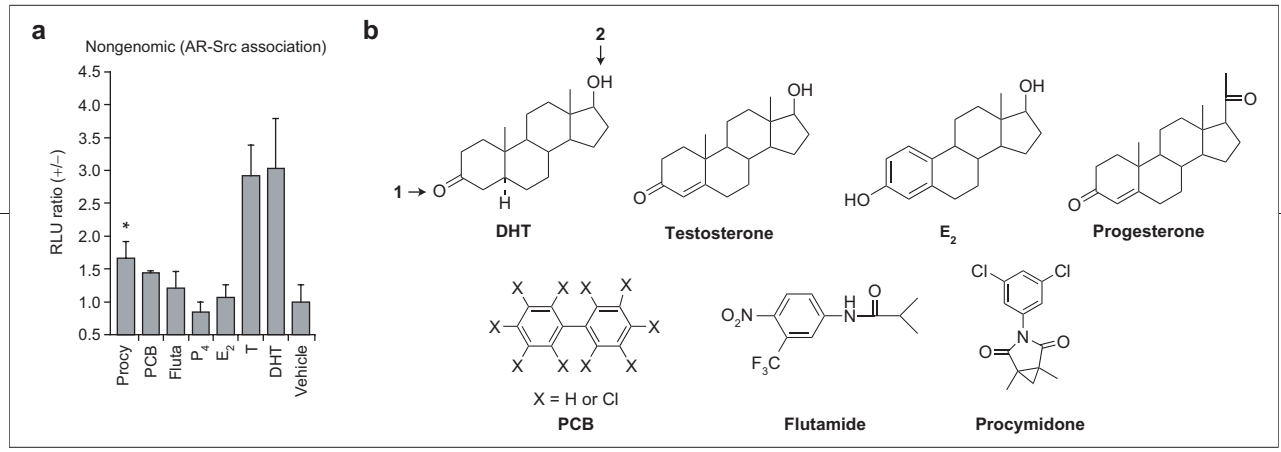


**Figure 4. Nongenomic activities of various ligands on AR–Src association. a) The nongenomic activities of ligands were estimated with pAR-Fn10 and pSrc-Fc5. The MCF-7 cells carrying pAR-Fn10 and pSrc-Fc5 were stimulated with $10^{-5}$ M DHT, testosterone (T), $E_2$, progesterone, PCB (Aroclor 1254), flutamide (fluta), and procymidone (procy), respectively ($n = 3$). The luminescence intensities induced by each ligand were compared with those from mock-stimulated cells ($n = 3$). The asterisk (*) shows the selective activity of procymidone for the AR–Src interaction in the nongenomic pathway of AR. b) Chemical structures of steroids and synthetic chemicals, used in the present study. Arrows 1 and 2 show C3-keto and C17β-hydroxy groups, respectively.**

(iii) no variation was observed in the expressed level of the fusion proteins.

**Determination of Dose-Dependent Hormonal Activities of Steroids Based on the AR–Src Association.** The hormonal activities of DHT and 17β-estradiol ($E_2$) were estimated on the basis of the AR–Src association (Figure 3, panel a). MCF-7 cells carrying pAR-Fn10 and pSrc-Fc5 were stimulated with differing concentrations of DHT or $E_2$, which ranged from 0 to $10^{-5}$ M. The luminescence intensities upon addition of DHT were dose-dependently increased from $10^{-10}$ M DHT and reached to a plateau at $\sim 10^{-8}$ M DHT. The detection limit and the 50% effective concentration ($EC_{50}$) in the DHT dose–response curve were found to be $\sim 10^{-10}$ and $\sim 10^{-9}$ M, respectively. On the other hand, $E_2$ did not induce any increase in the luminescence intensities with a concentration ranging from 0 to $10^{-5}$ M.

The higher luminescence intensities with DHT than with $E_2$ from the MCF-7 cells are explained as follows: (i) the recovery of FLuc activity indeed occurred agonist-dependently in the MCF-7 cells carrying pAR-Fn10 and pSrc-Fc5, and (ii) DHT is a more potent activator for the AR–Src association than $E_2$ in this nongenomic pathway of AR.

**Ligand-Dependent Kinetics of the AR–Src Association.** The ligand-dependent time course of the AR–Src association was determined in the presence or absence of $10^{-5}$ M DHT (Figure 3, panel b). A stimulation with $10^{-5}$ M DHT quickly increased the luminescence intensities, which reached a plateau 10 min after the stimulation (the half-luminescence time ($t_{1/2}$) is ~5 min). On the other hand, an addition of vehicle (0.1% (v/v) DMSO; final concentration) did not exhibit any increase in the luminescence intensity from the MCF-7 cells during the time period from 0 to 30 min. The results conclude that the present method provides an enough sensitivity and rapidness to detect ligand activities for protein–protein interactions in nongenomic pathways of AR. The DHT-induced AR–Src association occurs in 10 min, which is not only a net time of AR–Src interaction alone but also includes the time of split FLuc complementation as well.

**Inhibitory Effects of Androgen Antagonists on the AR–Src Association.** Flutamide and CPA are representative chemicals for nonsteroidal and steroidal androgen antagonists, respectively. The inhibitory effects of flutamide and CPA, respectively, on the AR–Src association were estimated (Figure 3, panel c). A $10^{-4}$ M concentration of flutamide decreased the luminescence intensities to 20% of those with $10^{-5}$ M DHT, and they were completely inhibited by $10^{-4}$ M CPA. The results demonstrate that CPA is a more potential antagonist for the AR–Src association than flutamide, and the AR–Src association is a ligand-controlled signaling pathway inhibited by androgen antagonists.

**Determination of the Ligand Selectivity of the Present Method.** The ligand selectivity of the present method was explored with several ligands, steroid hormones, and synthetic chemicals (Figure 4, panel a). Among steroids, DHT and testosterone (T) are known as full androgen agonists for the genomic action of AR. They induced the highest luminescence intensities among tested ligands. On the other hand, $E_2$ and progesterone are female sex hormones and are known to have partial agonistic activities for the genomic action of AR (*16*). They exhibited no significant activity for the AR–Src association. It is interesting that the absence of either C3-keto or C17β-hydroxy groups in the steroid backbone appears to eliminate the agonistic activities of $E_2$ and progesterone (Figure 4, panel b). On the other hand, synthetic chemicals such as procymidone and polychlorinated biphenyl (PCB; Aroclor 1254) showed weaker agonistic activities for the present AR–Src association. A $10^{-5}$ M concentration of procymidone and PCB in-

duced the luminescence intensities to 33% and 22%, respectively, of that with the same concentration of DHT.

The relative luminescence intensities from the MCF-7 cells after being stimulated with $10^{-5}$ M steroids or synthetic chemicals were as follows in decreasing order: DHT = T >> procymidone > PCB > flutamide > $E_2$ > progesterone. This ligand selectivity based on the nongenomic pathway of steroid signaling was compared with that of the genomic pathway due to the nuclear transport of AR (Figure 4, panel a and Supplementary Figure 1). The selectivity in the genomic pathway was obtained with the previous indicators developed for determining nuclear trafficking of AR, pcRDn-NLS, and pcDRc-AR (*16*). The comparison revealed that the relative androgenic activities of ligands on the AR–Src associations were not always parallel with those of ligands on the nuclear transport of AR. The difference in the ligand selectivity between the two methods was especially clear in the case of progesterone and $E_2$, that is, these compounds exhibited a considerable androgenic activity on the nuclear trafficking of AR but not on the AR–Src association.

The classical mechanism of NR action involves ligand binding to receptors, after which the receptors dimerize and bind to DNA. Ligands regulate gene transcription by interacting with intracellular NRs, which act as ligand-dependent transcription factors. The ligand-induced gene expression is regulated at the protein level some hours after stimulation with the ligand (genomic pathway) (*1–4*). Such a ligand capable of activating an NR for protein synthesis has been called "agonist" in the genomic pathway, whereas an antagonist is a substance that binds to a NR and blocks an action of the ligand without eliciting a biological response.

On the other hand, some other effects of sex hormones occur much faster than can be explained by a simple genomic pathway. They have been called a nongenomic pathway in steroid signaling. These nongenomic actions of steroids are characterized to occur in the cytosolic compartment and lead to activation of protein kinase cascades near the plasma membrane (Figure 1, panel a) (*1–4*). Recent studies revealed that various nongenomic pathways of steroid hormone signaling are related with human health and metabolism, for instance, the cardiovascular systems, central nervous systems, infertility, and electrolyte abnormalities (*19–22*). Differing from the genomic action, the nongenomic action does not require coactivators for transcription and is characterized by their rapid onset of the action (within several minutes) (*1–4*). However, it is difficult to define agonist and antagonist in nongenomic pathways of NRs, because a variety of potential mechanisms are involved in rapid steroid action. Many ligands may activate the intramolecular conformational changes and/or phosphorylation of NRs in the initial step of nongenomic pathways of steroid signaling. Here, we assume the agonist and antagonist in the nongenomic pathway be the ligand that induces and blocks the AR–Src binding, respectively.

We studied herein a system of the androgen-induced association of AR with a tyrosine kinase Src, a known target for anticancer drugs. The present study provides a look at the agonistic and antagonistic activities of ligands based on AR–Src binding as one of nongenomic pathways in steroid signaling. The activities were visualized in the form of luminescence intensities by the present probe. The relative luminescence intensities were taken as a measure of ligand selectivity of the probe.

We earlier determined the agonist selectivity of steroids based on a nuclear transport of AR in the genomic pathway (*16*). The method was developed for monitoring the nuclear trafficking of AR *in vitro* and *in vivo*. The targeting AR fused to the C-terminal half of *Renilla* luciferase (RLuc) is expressed in mammalian cells. If AR translocates into the nucleus, the RLuc moiety meets the N-terminal half of RLuc, and full-length RLuc is reconstituted by protein splicing. The bioluminescence intensities from the restored RLuc were determined as a measure of the genomic activities of ligands. The selectivity order is as follows in decreasing order: DHT > T > $E_2$ > progesterone > PCB ≥ flutamide ≥ procymidone (*16*). $E_2$ and progesterone are agonists for both androgen and estrogen signaling pathways because they activate not only ER but also AR (*23*). PCB, flutamide, and procymidone are known synthetic chemicals. Therefore, we compared the agonist selectivity of genomic and nongenomic signaling pathways of AR. The comparison of the agonist selectivity revealed that the full agonists are all potential activators for both the genomic and nongenomic pathways, but $E_2$, progesterone, and some synthetic chemicals exhibited a biased activity for either genomic or nongenomic pathways (Figure 4, panel a and Supplementary Figure 1). DHT and T showed full agonistic activities to both the genomic and nongenomic signaling pathways, whereas procymidone

showed an androgenic activity for only the nongenomic pathway. On the other hand, female sex hormones $E_2$ and progesterone exhibited agonistic activities to only the genomic pathway and not to the AR–Src interaction in the nongenomic pathway.

These characteristic ligand selectivities indicate that AR has its mechanisms to discriminate full agonists from female sex hormones and synthetic chemicals. A possible mechanism for AR to discriminate ligands in both genomic and nongenomic pathways is as follows. DHT, T, $E_2$, and progesterone are all steroid sex hormones and bind with AR. Upon binding to AR, steroids recognize distinct regions of the ligand binding domain of androgen receptor (AR LBD), which would result in different conformational changes in AR. The differences in the conformational change by the ligands subsequently may reflect in different luminescence intensities from the cells in both systems, genomic and nongenomic pathways. We reasoned that $E_2$ and progesterone induced different AR conformational changes from androgens, which accelerated a distinct magnitude of AR–Src binding in the nongenomic pathway of AR.

The characteristic ligand selectivity in the present method is also explained as follows. Although ligands activate both genomic and nongenomic signaling pathways of AR, the ligand activities are transduced downstream through different signaling steps. For nuclear transport in the genomic pathway, AR dimerizes and recruits many coactivators and coregulators (*2*). On the other hand, AR does not need to be dimerized in the nongenomic signaling. Ligand-activated AR experiences an intramolecular conformational change, which is the same as the genomic pathway. However, the activated monomeric AR then forms a complex with both Src and ER on the PM, where a short proline-rich region (residues 371–422) of the monomeric AR interacts with Src (*6*). The differences in the mechanisms between genomic and nongenomic pathways of AR may cause the distinct selectivity of the ligands.

Procymidone, PCB, and flutamide are known as nonsteroidal androgen antagonists. Their antiandrogenic activities have been determined on the basis of the AR actions in genomic pathways (*2, 16*). The three synthetic compounds were found to exhibit a weak agonistic activity on the AR–Src association, although they are androgen antagonists in the genomic pathway. Previously, antagonists have been categorized into two groups on the basis of their activity to the genomic pathway: one is "pure antagonist" and the other is "partial antagonist" (*24, 25*).

Flutamide is known to provide a clinical effect in the treatment of prostate cancer (*24*), whereas procymidone was originally introduced as a fungicide (*26*). Here we demonstrated that (i) flutamide and PCB exhibit weak agonistic activities in the both genomic and nongenomic pathways (Figure 4, panel a), and (ii) procymidone, however, has an activity only for the AR–Src association in the nongenomic pathway but not for the translocation of AR into the nucleus in the previous studies on the genomic pathway of AR (*2, 16*).

Flutamide and CPA are representative nonsteroidal and steroidal prostate cancer drugs, respectively. Their antagonistic activities on the nongenomic pathway of AR were evaluated with the present probe. A comparison of the antagonistic activity of flutamide with that of CPA revealed that CPA is a more efficient inhibitor than flutamide for the AR–Src association in the nongenomic AR signaling pathway (Figure 3, panel c). We thus concluded that CPA is an effective inhibitor for the nongenomic AR–Src signaling pathway of AR contributing to prostate cancer growth.

**Conclusion.** Androgen induces cell proliferation by increasing the kinase activity of Src, which is a well-known target for anticancer drugs. We constructed a pair of genetically encoded bioluminescent indicators for determining the association of AR with Src in human breast cancer-derived MCF-7 cells. The indicator was utilized for determining the agonistic and antagonistic activities of steroids and chemicals on the nongenomic pathway of AR. The relative activities of androgen agonists and antagonists on the AR–Src association were compared with those based on the nuclear transport of AR in the genomic pathway of AR. The indicators, pcRDn-NLS and pcDRc-AR, for estimating nuclear trafficking of AR have been reported previously (*16*). The comparison revealed that (i) full agonists such as DHT and T have activities for AR action in both the genomic and nongenomic pathways in AR signaling, (ii) female sex hormones $E_2$ and progesterone activated only the genomic pathway of AR, and (iii) the fungicide procymidone exerted a specific activity only for the nongenomic pathway of AR but not for the genomic pathway of AR. We also demonstrated that CPA is a more effective inhibitor for AR–Src interactions than flutamide. The present approach was found feasible for discriminating the activities of ligands on nongenomic

pathways of NRs in a cytosolic compartment of mammalian cells. The cytosolic AR–Src interactions cannot be determined with conventional reporter gene assays including yeast or mammalian two-hybrid systems.

## METHODS

**Construction of Plasmids.** Eight kinds of plasmids were constructed (Table 1). The plasmids containing cDNA of full-length AR were prepared as follows. The cDNA fragments encoding the N- and C-terminal fragments of FLuc (FLuc-N, 1–416 aa and FLuc-C, 417–550 aa) and full-length human AR (1–920 aa) were generated from each template using corresponding primers to introduce the unique enzyme sites as shown in Figure 1, panel c. DNA-modifying enzymes of the highest available purity were from Takara Bio Inc. The cDNAs were subcloned into the expression vector, pcDNA 3.1(+) vector (Invitrogen). The sequences of the cDNAs were confirmed with a BigDye Terminators v1.1 cycle sequencing kit and a genetic sequencer (ABI PRISM 310 Genetic Analyzer, PE Biosystems). The plasmids were named pAR-Fn5, pAR-Fn10, pAR-Fc5, and pAR-Fc10 according to the linker length and the type of FLuc fragment.

To construct the plasmids with Src, the cDNA fragments encoding Src (1–536 aa) and the N- and C-terminal fragments of FLuc were amplified from each template by polymerase chain reaction using corresponding primers to introduce the unique enzyme sites as shown in Figure 1, panel c. The cDNAs were then cloned in the pcDNA 3.1(+) vectors. The sequences of the cDNAs were also determined to ensure fidelity with a BigDye Terminators v1.1 cycle sequencing kit and the genetic sequencer. The plasmids were named pSrc-Fn5, pSrc-Fn10, pSrc-Fc5, and pSrc-Fc10 according to the linker length and the type of FLuc fragment. Optimal combinations of the present plasmids carrying AR and Src were preliminarily examined with the following transfection protocol (Figure 2, panel a).

**Cell Culture and Transfection.** MCF-7 cells derived from human breast cancer were cultured in a modified Eagle's medium (Sigma) supplemented with 10% (v/v) steroid-free fetal bovine serum (charcoal-extracted), non-essential amino acids (Gibco), and sodium pyruvate (Gibco) in addition to 1% (v/v) penicillin/streptomycin (Gibco) at 37 °C in 5% (v/v) $CO_2$. The cells were seeded in 6- or 12-well culture plates, transiently transfected with 2 or 1 μg of constructed plasmids per well using a transfection reagent, *Trans*IT-LT1 (Mirus), and incubated at 37 °C in 5% (v/v) $CO_2$ before experiments. The transfection reagent provides about 8% (n/n) of transfection efficiency for MCF-7 cells when incubated for 24 h.

**Western Blotting.** Expressions of pAR-Fn10 and pSrc-Fc5 were examined with Western blot (Figure 2, panel b). MCF-7 cells were cultured on 6-well culture dishes, transiently cotransfected with pAR-Fn10 and pSrc-Fc5, and incubated at 37 °C in 5% (v/v) $CO_2$ for 16 h. The cells were washed with PBS and lysed in a 200 μL of lysis buffer (1% (w/v) SDS, 10% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.001% (w/v) Bromophenol Blue, 50 mM Tris-HCl, pH 6.8). Each 7 μL sample was electrophoresed in a 10% (w/v) polyacrylamide gel, transferred to nitrocellulose membranes, and blotted with mouse anti-AR antibody (Santa Cruz), mouse anti-V5 epitope antibody (Invitrogen), or mouse anti-β-actin antibody (Sigma). The blots were incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) and visualized by an ECL Western blotting detection system (GE Healthcare) and a luminescence image analyzer (LAS-1000, Fuji Film). As a reference for the amounts of the electrophoresed proteins, β-actin was stained with its specific antibody.

**Determination of the Dose-Dependent Activities of Steroids on the AR–Src Association.** The luminescence intensities caused by a ligand-activated association of AR with Src were taken as a measure of the activity of the ligand (Figure 3, panel a). For the study, MCF-7 cells in 12-well plates were transiently cotransfected with pAR-Fn10 and pSrc-Fc5 and incubated at 37 °C in 5% (v/v) $CO_2$ for 18 h. The cells were stimulated with varying concentrations of DHT or $E_2$ for 20 min. The recovered FLuc activities by the association between AR and Src were estimated with a luminescence assay kit, Bright-Glo (Promega), according to the manufacturer's manual. The brief procedure of the Bright-Glo assay kit is as follows. The MCF-7 cells on the 12-well plates were washed once with PBS, and 80 μL of the D-luciferin solution in the kit was added to each well of the plates. Three minutes after the solution was injected, the luminescence intensities from each cell lysate were recorded with a luminometer (MiniLumat LB9506; Berthold). The amounts of proteins subjected were sequentially determined with a protein assay reagent (Bio-Rad) for the luminescence normalization as follows. The firefly luminescence intensities divided by the injected amount of the cell lysates were expressed as relative luminescence unit (RLU) ratio (+/−), that is, RLU (+)/ RLU (−), where RLU (+) and RLU (−) are the luminescence intensities with 1 μg of cell lysate after the cells were incubated with and without a ligand, respectively; the RLU (an amplified value of photon counts) is the unit of raw data from the luminometer. The RLU per 1 μg from ligand-stimulated cells was divided by that from mock-stimulated cells. The RLU from mock-transfected cells was nearly zero.

**Time Course.** Ligand-induced kinetics of the luminescence intensities was determined in the presence or absence of DHT (Figure 3, panel b). MCF-7 cells were transiently cotransfected with pAR-Fn10 and pSrc-Fc5 and incubated at 37 °C in 5% (v/v) $CO_2$ for 18 h. They were then stimulated with vehicle (0.1% (v/v) DMSO; final concentration) or $10^{-5}$ M DHT. At 0, 5, 10, 20, and 30 min after the ligand stimulation, the cells were harvested, and the respective luminescence intensities were developed with the Bright-Glo assay kit and determined with the luminometer at RT.

**Inhibition Study.** Inhibitory effects of flutamide and CPA on the AR–Src association were determined with the MCF-7 cells carrying pAR-Fn10 and pSrc-Fc5 (Figure 3, panel c). The MCF-7 cells were transiently cotransfected with pAR-Fn10 and pSrc-Fc5 and incubated at 37 °C in 5% (v/v) $CO_2$ for 18 h. The cells were first stimulated with $10^{-4}$ M flutamide or CPA for 20 min. The cells were then stimulated additionally with $10^{-5}$ M DHT for 20 min. The luminescence intensities of each well were developed with the Bright-Glo assay kit and determined with the luminometer at RT.

**Determination of Relative Activities of Steroids and Synthetic Chemicals at $10^{-5}$ M.** Relative activities of steroid hormones and synthetic chemicals on the AR–Src association at a fixed concentration were estimated with the MCF-7 cells carrying pAR-Fn10 and pSrc-Fc5 (Figure 4, panel a). The MCF-7 cells were transiently cotransfected with pAR-Fn10 and pSrc-Fc5 and incubated at 37 °C in 5% (v/v) $CO_2$ for 18 h. The cells were stimulated with $10^{-5}$ M steroids or synthetic chemicals shown in Figure 4, panel b for 20 min, and then the developed luminescence intensities were determined with the luminometer at RT.

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*Supporting Information Available:* This material is available free of charge *via* the Internet.

## REFERENCES


1. Norman, A. W., Mizwicki, M. T., and Norman, D. P. G. (2004) Steroid-hormone rapid actions, membrane receptors and a conformational ensemble model, *Nat. Rev. Drug Discovery 3*, 27–41.
2. Roy, A. K., Tyagi, R. K., Song, C. S., Lavrovsky, Y., Ahn, S. C., Oh, T. S., and Chatterjee, B. (2001) Androgen receptor: Structural domains and functional dynamics after ligand-receptor interaction, *Ann. N. Y. Acad. Sci. 949*, 44–57.
3. Heinlein, C. A., and Chang, C. S. (2002) The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions, *Mol. Endocrinol. 16*, 2181–2187.
4. Losel, R. M., Falkenstein, E., Feuring, M., Schultz, A., Tillmann, H. C., Rossol-Haseroth, K., and Wehling, M. (2003) Nongenomic steroid action: Controversies, questions, and answers, *Physiol. Rev. 83*, 965–1016.
5. Lee, H. J., and Chang, C. (2003) Recent advances in androgen receptor action, *Cell. Mol. Life Sci. 60*, 1613–1622.
6. Migliaccio, A., Castoria, G., Di Domenico, M., de Falco, A., Bilancio, A., Lombardi, M., Barone, M. V., Ametrano, D., Zannini, M. S., Abbondanza, C., and Auricchio, F. (2000) Steroid-induced androgen receptor-oestradiol receptor β-Src complex triggers prostate cancer cell proliferation, *EMBO J. 19*, 5406–5417.
7. Kousteni, S., Bellido, T., Plotkin, L. I., O'Brien, C. A., Bodenner, D. L., Han, L., Han, K., DiGregorio, G. B., Katzenellenbogen, J. A., Katzenellenbogen, B. S., Roberson, P. K., Weinstein, R. S., Jilka, R. L., and Manolagas, S. C. (2001) Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: Dissociation from transcriptional activity, *Cell 104*, 719–730.
8. Ballare, C., Uhrig, M., Bechtold, T., Sancho, E., Di Domenico, M., Migliaccio, A., Auricchio, F., and Beato, M. (2003) Two domains of the progesterone receptor interact with the estrogen receptor and are required for progesterone activation of the *c*-Src/Erk pathway in mammalian cells, *Mol. Cell. Biol. 23*, 1994–2008.
9. Yeatman, T. J. (2004) A renaissance for SRC, *Nat. Rev. Cancer 4*, 470–480.
10. Ishizawar, R., and Parsons, S. J. (2004) *c*-Src and cooperating partners in human cancer, *Cancer Cell 6*, 209–214.
11. Paulmurugan, R., Umezawa, Y., and Gambhir, S. S. (2002) Noninvasive imaging of protein-protein interactions in living subjects by using reporter protein complementation and reconstitution strategies, *Proc. Natl. Acad. Sci. U. S. A. 99*, 15608–15613.
12. Kaihara, A., Kawai, Y., Sato, M., Ozawa, T., and Umezawa, Y. (2003) Locating a protein-protein interaction in living cells via split Renilla luciferase complementation, *Anal. Chem. 75*, 4176–4181.
13. Luker, K. E., Smith, M. C., Luker, G. D., Gammon, S. T., Piwnica-Worms, H., and Piwnica-Worms, D. (2004) Kinetics of regulated protein-protein interactions revealed with firefly luciferase complementation imaging in cells and living animals, *Proc. Natl. Acad. Sci. U. S. A. 101*, 12288–12293.
14. Remy, I., and Michnick, S. W. (2006) A highly sensitive protein-protein interaction assay based on Gaussia luciferase, *Nat. Methods 3*, 977–979.
15. Pelletier, J. N., Campbell-Valois, F. X., and Michnick, S. W. (1998) Oligomerization domain-directed reassembly of active dihydrofolate reductase from rationally designed fragments, *Proc. Natl. Acad. Sci. U. S. A. 95*, 12141–12146.
16. Kim, S. B., Ozawa, T., Watanabe, S., and Umezawa, Y. (2004) High-throughput sensing and noninvasive imaging of protein nuclear transport by using reconstitution of split Renilla luciferase, *Proc. Natl. Acad. Sci. U. S. A. 101*, 11542–11547.
17. Kim, S. B., Takao, R., Ozawa, T., and Umezawa, Y. (2005) Quantitative determination of protein nuclear transport induced by phosphorylation or by proteolysis, *Anal. Chem. 77*, 6928–6934.
18. Paulmurugan, R., and Gambhir, S. S. (2005) Firefly luciferase enzyme fragment complementation for imaging in cells and living animals, *Anal. Chem. 77*, 1295–1302.
19. Schmidt, B. M. W., Gerdes, D., Feuring, M., Falkenstein, E., Christ, M., and Wehling, M. (2000) Rapid, nongenomic steroid actions: A new age? *Front. Neuroendocrinol. 21*, 57–94.
20. Thomas, P., and Doughty, K. (2004) Disruption of rapid, nongenomic steroid actions by environmental chemicals: Interference with progestin stimulation of sperm motility in Atlantic croaker, *Environ. Sci. Technol. 38*, 6328–6332.
21. Rosano, G. M. C., Leonardo, F., Pagnotta, P., Pelliccia, F., Panina, G., Cerquetani, E., della Monica, P. L., Bonfigli, B., Volpe, M., and Chierchia, S. L. (1999) Acute anti-ischemic effect of testosterone in men with coronary artery disease, *Circulation 99*, 1666–1670.
22. Liu, P. Y., Death, A. K., and Handelsman, D. J. (2003) Androgens and cardiovascular disease, *Endocrinol. Rev. 24*, 313–340.
23. Tomura, A., Goto, K., Morinaga, H., Nomura, M., Okabe, T., Yanase, T., Takayanagi, R., and Nawata, H. (2001) The subnuclear three-dimensional image analysis of androgen receptor fused to green fluorescence protein, *J. Biol. Chem. 276*, 28395–28401.
24. Singh, S. M., Gauthier, S., and Labrie, F. (2000) Androgen receptor antagonists (antiandrogens): structure-activity relationships, *Curr. Med. Chem. 7*, 211–247.
25. Klein-Hitpass, L., Tsai, S. Y., Weigel, N. L., Allan, G. F., Riley, D., Rodriguez, R., Schrader, W. T., Tsai, M. J., and Omalley, B. W. (1990) The progesterone-receptor stimulates cell-free transcription by enhancing the formation of a stable preinitiation complex, *Cell 60*, 247–257.
26. Svechnikov, K., Supornsilchai, V., Strand, M. L., Wahlgren, A., Seidlova-Wuttke, D., Wuttke, W., and Soder, O. (2005) Influence of long-term dietary administration of procymidone, a fungicide with anti-androgenic effects, or the phytoestrogen genistein to rats on the pituitary-gonadal axis and Leydig cell steroidogenesis, *J. Endocrinol. 187*, 117–124.