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Estrogen-specific Target Site Identified by Progesterone-11α-hemisuccinate-(2-[^{125}I]-iodohistamine) in Mouse Brain Membranes

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Using photoaffinity labeling with the progesterone analogue, progesterone-11α-hemisuccinate-(2-[^{125}I]-iodohistamine) ([^{125}I]-his-PG), we identified and characterized a protein band of MW 29 kDa (p29) in mouse cerebellar membranes whose labeling is markedly inhibited by estrogens. Inhibition of the labeling was specific with respect to steroid structure. Labeling was strongly inhibited by estradiol-17β, estradiol-17α, and the anti-estrogen tamoxifen and the synthetic estrogen diethylstilbestrol. Other estrogens (estriol, estrone) were less effective and the steroids, dihydroandrosterone, androsterone and aldosterone were ineffective. Preincubation with estradiol-17α or estradiol-17β inhibited the labeling in a dose-dependent manner with IC_{50} values of 0.3 and 2.0 μM, respectively. The extent of labeling was three times as high in cerebellar membranes from males as from females. In males, labeling of cerebellar membranes was greater than that of cortex or limbic region. The labeling pattern of p29 was also different in membranes prepared from cerebellum, heart and liver. Moreover, PG enhanced the labeling of p29 in liver demonstrating a tissue-specific mode of interaction. The present results characterize p29 as a membrane-bound estrogen target site. Copyright © 1996 Elsevier Science Ltd.

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INTRODUCTION

Recent studies have shown that membrane-bound steroid receptors play important roles in brain and peripheral tissues [1, 2]. Certain steroids such as estradiol-17β (17β-E_2) and progesterone (PG) demonstrate rapid non-genomic effects that have been hypothesized to occur at the level of the membrane [1–3]. Direct application of 17β-E_2 increases the potassium conductance in amygdalar neurons [4] and rapidly potentiates the K^+-induced LH–RH release in hypothalamic slices [5]. 17β-E_2 also increases Ca^{2+}-dependent spiking activity and internal Ca^{2+} levels in a pituitary cell line (GH3) [6] and chicken granulosa cells obtained from preovulatory follicles [7], respectively. Progesterone (PG) mediates rapid responses such as enhancement of the Cl-currents induced by GABA and inhibition of the Cl-currents induced by glycine [8]. Other non-genomic effects include the modulation of the membrane levels of oxytocin and serotonin receptors by PG [9] and 17β-E_2 [10], respectively.

Although we have considerable knowledge about intracellular steroid receptors and their genomic actions [11], comparatively little is known about the membrane-bound steroid target sites. Direct binding assays have already identified [^{3}H]17β-E_2 and [^{3}H]PG receptors in pituitary [12] and brain membranes, respectively [13]. In a recent study, a PG-specific membrane protein was purified from digitonin-solubulized cerebellar membranes by using an agarose-bovine serum albumin-3-PG column [14].

We have recently reported the use of an ^{125}I-photoaffinity probe, progesterone-11α-hemisuccinate-(2-[^{125}I] iodohistamine) ([^{125}I]-his-PG) to label progestin binding proteins in brain membranes [15]. Photoaffinity labeling with ^{3}H-probes has historically been used to label PG membrane target sites [16, 17]. We chose the ^{125}I-probe in order to achieve higher resolution. We described a rapid and sensitive method and identified four PG binding proteins (bands 1–4) in cerebellar membranes with apparent molecular weights 64, 60, 54 and 29 kDa (p64–p29) [15]. The extent of the

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inhibition of labeling for p64 and p29 was specific with respect to steroid structure. The only steroids that substantially inhibited the labeling were PG and 3α for p64 and 17β-E_2 for p29. Other steroids, that included testosterone, corticosterone and $3\alpha5\alpha$ pregnanolone, were far less effective. The inhibition of photoaffinity labeling with unlabeled steroids suggests that PG and 3α are specific for p64 and 17β-E_2 is specific for p29. It was also demonstrated that all four bands were membrane bound. Treatments of the labeled bands with hypotonic or high salt buffers left the pattern and intensity of the labeled bands essentially unchanged. The unlabeled form of the ^{125}I-probe is not commercially available. Therefore, its dissociation constant and biological activity were not determined.

Here we evaluate the specificity of the labeling of p29 with estrogens including 17β-E_2, estradiol-17α (17α-E_2), estriol (E_3), estrogen (E_1), the antiestrogen tamoxifen (Tam), the synthetic estrogen diethylstilbestrol (Des) as well as progesterone (PG), aldosterone (Ald) and the androgens, androsterone (And) and dihydroandrosterone (DHA). Possible physiological and pharmacological roles for this protein are sought by comparing the extent and specificity of its labeling in membranes from different tissues including heart and liver, and brain membranes from male and female mice.

MATERIALS AND METHODS

Mice (albino CD-1; 18–20 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA). Steroids and other reagents were obtained from Sigma (St Louis, MO). Progesterone-11α-hemisuccinate (2-^{125}I] iodohistamine) ([^{125}I]-his-PG; 2000 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). The purity of [^{125}I]-his-PG was checked by thin layer chromatography (TLC) [18]. Autoradiography of the TLC plates showed a single peak.

Membranes

Mice were sacrificed by decapitation following anesthesia with carbon dioxide. Male mice were used unless specified otherwise. The brain regions (cerebellum, limbic region, cortex), heart and liver were separated and homogenized in a 0.32 M sucrose buffer (10 mM HEPES, pH 7.45) (5% w/v). Glass–glass and glass–teflon homogenizers were used for brain, heart and liver, respectively. After centrifugation at 2000***g*** for 15 min the supernatant was saved and the remaining pellet was rehomogenized and centrifuged. The resulting supernatants were combined and centrifuged at 30 000***g*** for 30 min (Beckman J2-21; Palo Alto, CA). The resulting pellet was resuspended in the phosphate buffer (50 mM Na/K phosphate, 200 mM NaCl, pH 7.45) in a final concentration of 6–10 mg protein/ml. Homogenization and centrifugations were done at 0–4°C. Protein was measured using bovine serum albumin as the standard [19].

Dissections were carried out on ice. An atlas of the mouse brain was used for a guide [20]. The cortices were removed and then the cerebellum. The remaining tissue was trimmed with a sharp razor blade. Tissue caudal to the midbrain and rostral to the olfactory tubercles was removed. The remaining tissue was termed the limbic region.

Photoaffinity labeling

[^{125}I]-his-PG (0.5 pmol) in methanol:water (9:1) was placed in a 7 ml glass vial (Research Products International, Mount Prospect, IL) and evaporated to dryness (in a water bath (70°C) under mild air flow). Then 260 μl of phosphate buffer containing dimethyl sulfoxide (1%) and ethanol (0.2%) was added and vortexed. Then membranes (1 mg/740 μl LB) were added and vortexed. This mixture was incubated for 30 min at room temperature in a dark container. The final concentration of [^{125}I]-his-PG was 0.5 nM. This constituted the [^{125}I]-his-PG binding sample.

Inhibition of labeling

Steroids, Des and Tam were added in DMSO (1 μl) to 100 μl of [^{125}I]-his-PG binding sample. Samples were vortexed and incubated for 30 min in the dark at room temperature. UV-irradiations were carried out at 254 nM with a model UVG-11 lamp (UVP, Inc., San Gabriel, CA) for 30 min/4°C. The distance between the lamp and sample was 7 cm. Then 7 μl of DTT (15.5 mg/ml) was added as a scavenger.

Gel electrophoresis and quantitation

Membrane proteins were resolved by SDS–PAGE in a 16% acrylamide (0.11% BIS) separating gel (1.5 mM) [21]. Routinely, 64 μg of protein were applied to a single lane, except in Fig. 4, 8O μg was used. The extent of labeling was determined by one of the following methods: (i) Phosphorimager (Molecular Dynamics, Sunnyvale, CA) (Figs 1, 3 and 4). The dried gels were exposed to phosphorimage screens for 4–6 days and the image was printed using the grey scale after background subtraction. The data were quantitated by ImageQuant Software; (ii) Cut and count (Fig. 2). Bands were cut out from destained gels and counted in a gamma counter (Micromedic 4/200, ICN Micromedic Systems, Inc., Huntsville, AL). Cpm was demonstrated to be linear ($r = 0.9$) over the labeling range for p29.

Statistics

The Student's t-test was used to evaluate the significance of the difference between means [22]. In Fig. 2, the following four-parameter logistic function was used to fit the data

$$f = (a - d)/[1 + (x/c)^b] + d,$$

where a is the maximum value, b is a slope parameter, c is the value at the inflexion point and d is the minimum

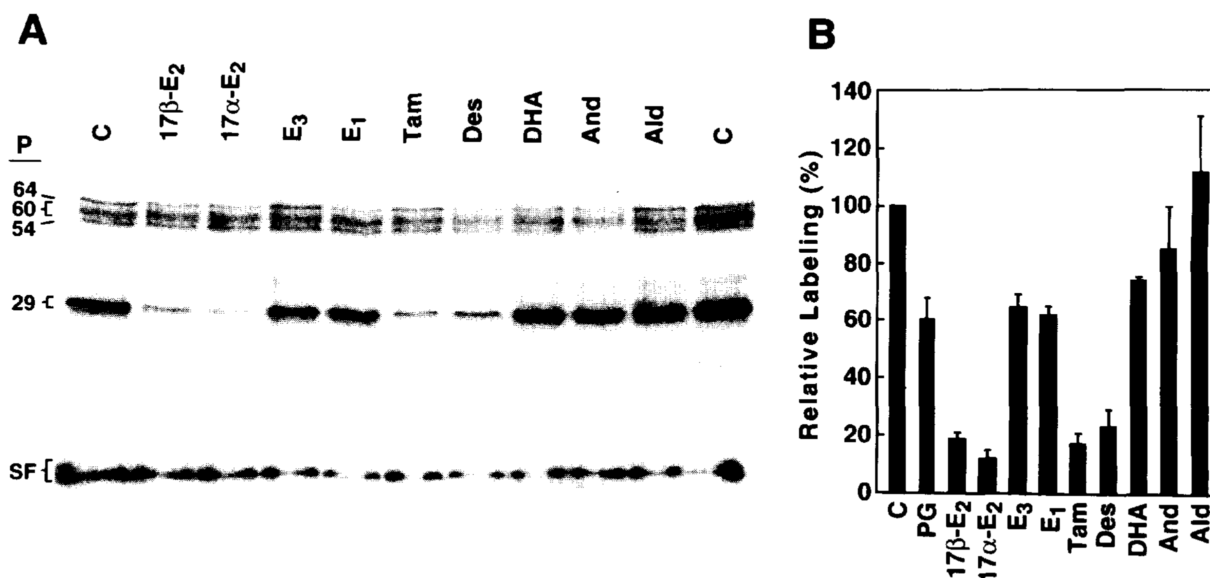

Fig. 1. Specific interaction between p29 and estrogens. (A) The autoradiogram shows the labeling profile. (B) The graph shows the corresponding values for p29 as determined by phosphorimage (ImageQuant). Membranes were incubated with [^{125}I]-his-PG in the absence (C) and presence of steroids (10 μM) including 17β-E$_2$, estradiol-17β; 17α-E$_2$, estradiol-17α; E$_3$, estriol; E$_1$, estrone; Tam, tamoxifen; Des, diethylstilbestrol; DHA, dihydroandrosterone; And, Androsterone; Ald, aldosterone. Samples (64 μg) were applied to SDS–PAGE. [^{125}I]-his-PG labeled proteins are shown as p64–p29 (64–29 kDa). SF is the solvent front. The data were normalized with (C) set equal to 100. The value for PG (progesterone) was obtained from separate gels. Bar heights show means $\pm$ SEM ($n = 3$).

value (Sigma Plot, Jandel Scientific, Corte Madera, CA).

RESULTS

Specificity of labeling

The effect of estrogens, other steroids and an anti-estrogen on p29 are shown in Fig. 1. The extent of labeling was markedly inhibited by 17β-E$_2$ (84%), 17α-E$_2$ (90%), Des (75%), as well as the estrogen antagonist Tam (80%) (Fig. 1B). E$_1$, E$_3$, PG, And, DHA, and Ald were less effective or ineffective. Because conditions were chosen to favor the quantitation of p29, p54–64 were not adequately resolved for quantitative analysis. However, the lack of inhibition by 17β-E$_2$ and 17α-E$_2$ is apparent for these bands (Fig. 1A).

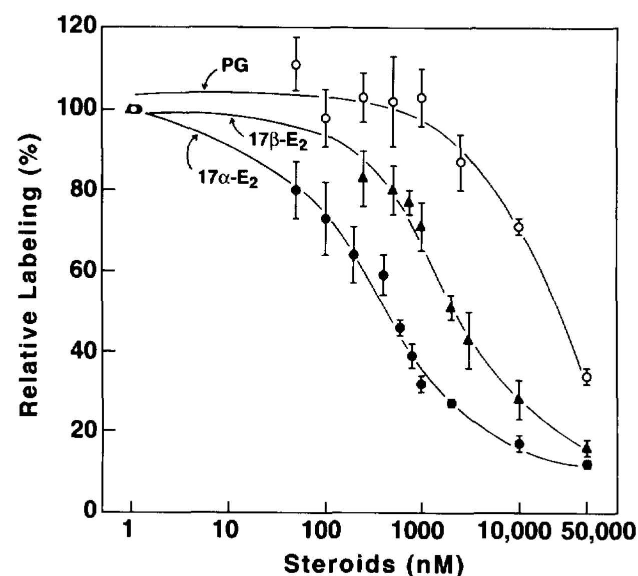

Fig. 2. Dose-dependent inhibition by estadiol 17α and estradiol 17β. Relative binding of [^{125}I]-his-PG as a function of PG, 17α-E$_2$ or 17β-E$_2$ (0–50 μM) was shown. Samples (64 μg) were applied to SDS–PAGE and p29 were cut from the gels and counted in a gamma counter ($n = 5$, mean $\pm$ SEM).

Dose-dependent inhibition by 17β-E$_2$ and 17α

Membranes were labeled in the presence of increasing concentrations of 17β-E$_2$ and 17α-E$_2$ (Fig. 2). The extent of p29 labeling was determined by cutting and counting the gel pieces. The data were fitted by a four parameter logistic function. The extent of labeling was inhibited in a dose-dependent manner. The IC$_{50}$ values were estimated from the effective-inhibition range and were found to be 0.3 μM for 17α-E$_2$ and 2.0 μM for 17β-E$_2$ (Fig. 2). Data for PG (IC$_{50}$ 20 μM) is from Bukusoglu and Krieger [15] and is included for the purpose of comparison.

Gender differences

The extent of labeling of p29 was compared in brain membranes from male and female mice (Fig. 3). In membranes prepared from male brain, the extent of labeling was highest for cerebellum (cer), intermediate for limbic region (lim) and lowest for cortex (cor) (Fig. 3).

Levels (male) were significantly lower in cor than in cer ($P < 0.05$). In membranes prepared from female

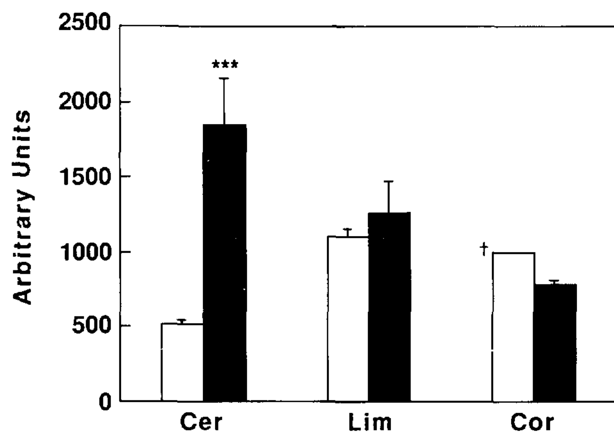

Fig. 3. Gender differences for p29. Membranes were prepared from female (white) and male (black) brain regions; cer, cerebellum; lim, limbic region; cor, cortex. The graph shows the extent of labeling for p29. The data were normalized with female-cortex (†) set equal to 1000. Bar heights are means ± SEM ($n = 4$). ***$P < 0.001$.

brain, the extent of labeling was not significantly different for lim and cor (Fig. 3). Labeling in the cer was the lowest. Levels were significantly higher in lim than in cer ($P < 0.01$). Levels were significantly ($P < 0.001$) higher in male than in female cer (3.5 times as high). Labeling of other regions was not significantly different in males and females.

Tissue distribution of p29

Labeling patterns of the heart and liver were compared to those in cer. Membranes were labeled with [^{125}I]-his-PG in the absence (C, control) or presence of PG, and 17β-E$_2$ (10 μM) (Fig. 4A). The labeling profile was different in each tissue (Fig. 4A). In cer, as previously shown, the four proteins (p29–p64) were observed. However, in heart and liver, with the exception of p29, [^{125}I]-his-PG labeled similar but different sets of bands.

In heart, the extent of labeling was much lower than in cer: p60 was absent, p54 was diffuse and p64 had a slightly different mobility from p64 in cer. In liver, the extent of labeling was markedly higher (Fig. 4A). There [^{125}I]-his-PG labeled four bands (1a, 3a, 3b and 4). Bands 1a and 3a showed similar mobilities to p64 and p54, respectively (Fig. 4A). As shown in Fig. 4B, the extent of labeling of p29 (control, white) was approximately 14 times as great in liver as in cer. Here PG is seen to enhance the extent of labeling compared to the control, whereas previously, in all instances, PG as well as every other steroid had been observed to inhibit the level of labeling of the control. In particular, 17β-E$_2$ was inhibitory and the percentage of inhibition was similar to that in cerebellum (Fig. 4B).

DISCUSSION

There are few in-depth studies of steroid binding sites in cell membranes. The identification of such sites has been hampered by their low copy number and steroid hydrophobicity [12], [23]. Here, photoaffinity labeling with [^{125}I]-his-PG in conjunction with SDS–PAGE

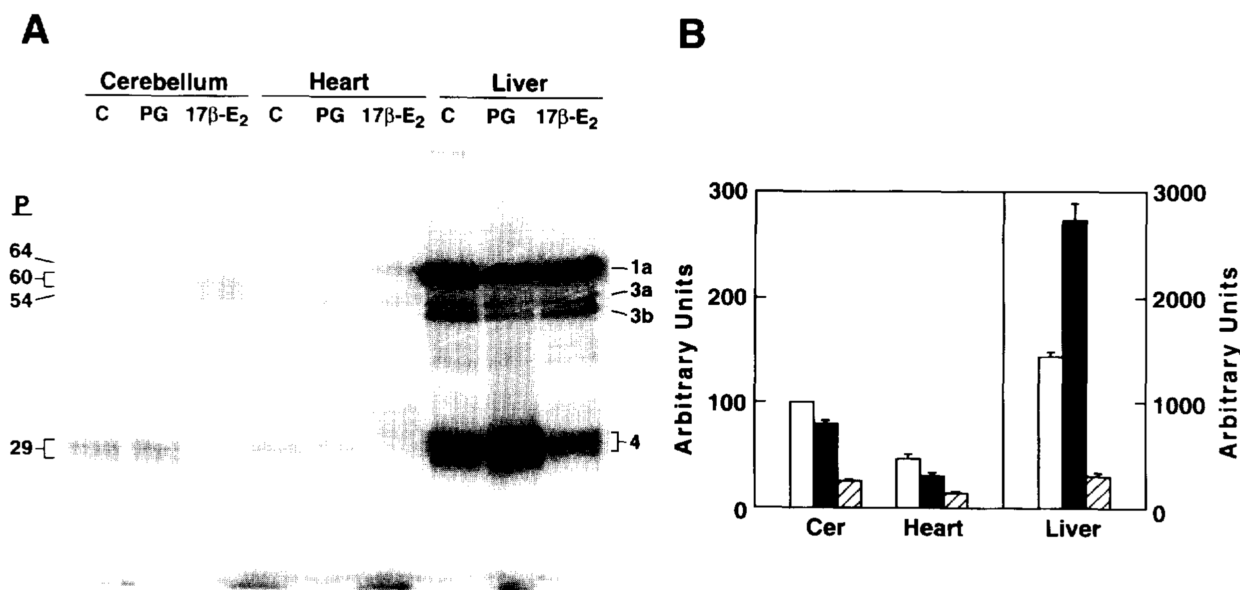

Fig. 4. Tissue specific labeling. (A) The labeling profile. Membranes were prepared from cerebellum (cer), heart and liver and incubated with [^{125}I]-his-PG in the absence (C, control) and presence of PG and 17β-E$_2$ (10 μM). Samples (80 μg) were applied to SDS–PAGE and exposed to phosphorimage. [^{125}I]-his-PG labeled cerebellar proteins are shown as p64–p29 (64–29 kDa). (B) The extent of labeling for p29 (ImageQuant). Bar heights for cer, liver are means ± SEM ($n = 3$); for heart bar heights are means ± range ($n = 2$). The data were normalized with the level of cer (white) set equal to 100. Absence of steroids (C, white); presence of PG (black); presence of estradiol (cross hatched). Arbitrary units: (i) cer, heart; (ii) liver.

overcame these difficulties and led to the identification of specific target sites for PG and 17β-E_2.

The present results show that the labeling of p29 by [^{125}I]-his-PG is highly specific with respect to steroid structure and indicate that this membrane-bound protein is an estrogen-specific target site. Among the 13 steroids tested here and previously [15], only 17β-E_2 and 17α-E_2 substantially (84–88%) inhibited the labeling. Similarly, the synthetic estrogen, Des, and the antiestrogen, Tam, were markedly inhibitory (77–83%). Other steroids including PG, And, DHA, Ald, $3\alpha5\alpha$ pregnanolone, pregnenolone sulfate, cholesterol, testosterone and corticosterone showed less or no effect. In Western blots full-length intracellular estrogen receptor shows an apparent molecular weight of 65 kD and a faint proteolytic fragment of 54 kD [24]. These molecular weights are similar to the [^{125}I]-his-PG labeled p64 and p54. However, contrary to the intracellular estrogen receptor, p64 and p54 were not sensitive to 17β-E_2 [15].

The relatively inactive 17α-E_2 has often been used to demonstrate the specificity of 17β-E_2 with respect to genomic activity [4, 6, 25]. However, for non-genomic steroid activities relative potencies of these two steroids are less well described. A potent activity for 17α-E_2 has already been shown in chicken granulosa cells obtained from preovulatory follicles [7]. Similarly, in this report 17α-E_2 exhibited a potent inhibitory effect upon labeling with [^{125}I]-his-PG. It is also of interest that Tam, which is used clinically at micromolar levels, was strongly inhibitory in the present system. In recent clinical studies, high doses of Tam were successfully used to inhibit the activity of P-glycoprotein (multidrug resistance) and protein kinase C in advanced refractory malignancy [26] and recurrent malignant glioma [27]. In these studies plasma and tumor Tam levels were in the range of 3–6 μM which are similar to the concentrations that were used here. Thus, under the present pharmacological conditions p29 might conceivably be a target for Tam.

The localization of p29 was specific but different in brain regions from males and females. The highest extent of labeling for this estrogen-specific target site was in male cerebellum, suggesting the possibility that the expression of p29 may be regulated by a sex steroid. The pattern of labeling was also different with respect to tissue. In the periphery, the extent of labeling was high in liver but relatively low in heart. The 14 times higher labeling of p29 in liver than cer may reflect higher levels of p29 in liver or a higher affinity for [^{125}I]-his-PG. The modulation by PG was also different in liver, where unlabeled PG enhanced the extent of labeling of p29, which was opposite to its effects in cer and in heart. Dose-response data would be required for a quantitative comparison. However the apparent high density of p29 in liver along with different PG modulation suggest the possibility of a localized function.

The concentrations that were found to be effective in inhibiting the labeling of p29 for 17β-E_2 (2 μM) and PG (20 μM) (Fig. 2) were pharmacological rather than physiological. To discover its physiological relevance, the functional properties of p29 need to be identified. The cloning and sequencing of p29 is the next step for this objective.

The specificity of [^{125}I]-his-PG labeling at p29 and steroid binding to the well-described intracellular estrogen receptor are distinct. 17α-E_2 and 17β-E_2 have opposite relative potencies at the two sites [24], [28] and PG lacks affinity for the intracellular 17β-E_2 receptor [29]. Interestingly, however, the rank order of potencies for 17β-E_2, diethylstilbestrol, E_1, E_3, androsterone and aldosterone at p29 parallels that for binding to the intracellular estradiol receptor [24], [28], [29]. Although the steroid affinities at p29 are far lower than those usually associated with the intracellular estrogen receptor, it is interesting to note the marked steroid specificity of the p29 site (Fig. 1), [15]. This specificity suggests that these estrogen target sites may share some structural homology.

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