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Correlation between PAP-dependent steroid binding activity and substrate specificity of mouse and human estrogen sulfotransferases

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Abstract

Estrogen sulfotransferase (EST) is a cytosolic enzyme that catalyzes the sulfoconjugation and inactivation of estrogens using 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as an activated sulfate donor. A finding of undetermined significance in the study of EST has been that the guinea pig EST is able to bind pregnenolone and estradiol with high affinity in the presence of PAP, the reaction by-product of the sulfate donor PAPS. This finding has raised the possibility that EST may have other physiological functions independent of its enzymatic activity as a sulfotransferase. To determine if the PAP-dependent steroid binding activity is a common property shared by other estrogen sulfotransferases, we have expressed the mouse and human EST in bacteria and used the purified protein to address this question. We found that, in the presence of PAP, both recombinant mouse and human EST were able to bind estradiol with high affinity but only the human EST was able to bind pregnenolone. In addition, we show that human but not the mouse EST was also able to bind dehydroepiandrosterone, a property that was not described for the guinea pig EST. Furthermore, we demonstrate that the promiscuity of human EST in steroid binding is mirrored by a correspondingly low substrate specificity in its enzymatic activity as a sulfotransferase. Reversely, the lack of stable binding of pregnenolone and dehydroepiandrosterone by the mouse EST is paralleled by a lack of sulfotransferase activity of this enzyme toward these two steroids. Mutagenesis of mouse EST within a domain critical for PAPS binding abolished both its sulfotransferase and PAP-dependent estrogen binding activity. These data suggest that stable binding of steroids such as pregnenolone or estrogen is not an independent property of estrogen sulfotransferases but rather is related to their catalytic activity.

1. Introduction

Estrogen sulfotransferase (EST) catalyzes the sulfoconjugation of estrogens at the 3-hydroxyl position using 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as an activated sulfate donor. EST is a member of the cytosolic sulfotransferase family of enzymes which are expressed in the mammalian liver where they are part of the drug metabolizing enzyme system [1–3]. Expression of EST has also been detected in estrogen target tissues such as the breast epithelium and a role for the enzyme in these tissues to act as a local estrogen modulator has been proposed [4–9]. In addition to the liver and primary estrogen target tissues, EST has been shown to be expressed abundantly in a number of steroidogenic tissues such as the bovine placenta [10], the guinea pig adrenal cortex [11] and the rodent and human testes [12,13]. We previously have established that within the mouse testis, the enzyme is localized selectively to the androgen-producing Leydig cells [13,14].

With regard to the potential physiological roles of EST in testicular Leydig cells [13,14], two attractive

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hypotheses could be formulated based on past studies. Firstly, similar to its role in the mammary epithelial cells, testicular EST may function as a local estrogen modulator since estrogen is both synthesized in the testis and required for its proper function. It is well accepted that testis is a major source of estrogen production in male animals and men [15–17]. Furthermore, recent studies with the estrogen receptor knockout mice have shown that estrogen plays an essential role in testicular function [18,19]. A second equally attractive hypothesis is that EST might function as a steroid binding protein to regulate steroidogenesis in Leydig cells, the placenta and the adrenal cortex. This concept originated from the fact that the guinea pig adrenal EST was purified, cloned and identified as a "pregnenolone-binding protein (PBP)" from the adrenal cortex [11]. Because pregnenolone is a common and rate-limiting precursor for various steroidogenic pathways, the possibility for a pregnenolone-binding protein to play some regulatory role in the steroidogenesis of the adrenal cortex is of significant interest [20–24]. Although the guinea pig protein was eventually cloned and identified as an EST, the significance of the pregnenolone-binding activity and its relationship to the estrogen sulfotransferase activity remain unknown. In addition to their ability to bind pregnenolone, both partially purified and recombinant guinea pig EST were shown to bind estradiol with high affinity [23,24]. This later finding has also raised the intriguing possibility that in the guinea pig adrenal cortex, EST may function more as a steroid-binding protein than as an enzyme [23].

In light of these findings with the guinea pig EST and the fact that EST is expressed prominently in testicular Leydig cells [13,14], we wondered if EST could function as a steroid-binding protein to modulate steroidogenesis in tissues such as the Leydig cells. A key question that relates to this hypothesis is whether the pregnenolone- and estradiol-binding activity described for the guinea pig EST is shared by other estrogen sulfotransferases. In this investigation, we have expressed and purified the mouse and human estrogen sulfotransferases and evaluated their ability to bind pregnenolone and other steroids. In addition, we have explored the mechanistic relationship between the steroid-binding and sulfotransferase activities of the two estrogen sulfotransferases.

2. Experimental procedures

2.1. Subcloning and mutagenesis of the mouse EST cDNA

The mouse EST cDNA has previously been cloned into the bacterial fusion protein expression vector pGEX-4T-3 (Pharmacia) at *Bam HI* and *Xho I* sites [12]. To generate a cDNA construct suitable for protein expression in eukaryotic cells, the mouse EST cDNA was removed from the pGEX-4T-3 vector by restriction digestion at *Bam HI* and *Xho I* sites and ligated into the eukaryotic expression vector pCDNA3 (Invitrogen, San Diego, CA) at the same sites. The resulting pCDNA3 construct was used for transfection into CHO cells and as a template for site-directed mutagenesis in the putative PAPS-binding domain of the mouse EST [12,25]. Two glycine residues (G259, G262) in this domain were simultaneously mutated to an alanine by the recombinant circle polymerase chain reaction method [26]. For this procedure, the QuikChange™ site-directed mutagene kit from Strategen (San Diego, CA) was used with the following two primers containing the desired nucleotide substitutions: 5′-CAT-GCG-AAA-GGG-AAT-TAT-AGG-AGA-CTG-GAA-GAA-C-3′ and 5′-GTT-CTT-CCA-GTC-TCC-TAT-AAT-TCC-CTT-TCG-CAT-G-3′. After rescuing the cDNA, the mutated sequences were confirmed by double-stranded DNA sequencing.

2.2. Expression of wild type and mutant mouse estrogen sulfotransferase

Wild-type mouse EST was expressed as a fusion protein in *E. coli* bacteria using the pGEX-4T-3 expression vector by following the previously described protocol [12]. Pure EST was obtained after thrombin digestion of the fusion protein and affinity chromatography [12]. In a separate experiment, the wild-type and the mutant enzymes were transiently expressed in CHO cells with the pCDNA3 expression vector. CHO-K1 cells (ATCC, Rockville, MD) were cultured in 100 mm culture dishes in MEM α-medium containing 10% fetal bovine serum, 2.5 mM HEPES, pH 7.5 and 2 mM glutamine. Cells were seeded at 60% confluence and transfected using Lipofectamine (Gibco/BRL, Grand Island, NY) the following day with 10 μg plasmid DNA of pCDNA3 containing the mouse EST cDNA. Cells were harvested 48 h later for steroid binding or enzyme activity assays.

2.3. Cloning and expression of the human EST

To amplify human EST cDNA by reverse-transcription polymerase chain reaction (RT-PCR), a sample of human liver total RNA was purchased from Clontech (Palo Alto, CA). First strand cDNA was synthesized and RT-PCR was performed as previously described [12]. The two oligonucleotide primers used for RT-PCR were 5′-CCC-GGG-ATC-AAC-TAA-ACA-GTG-TAC-C-3′ (upstream) and 5′-ACC-TTC-TTA-GAT-CTC-AGT-TCG-3′ (downstream).The upstream primer corresponds to the -22 to -4 region 5′ to the

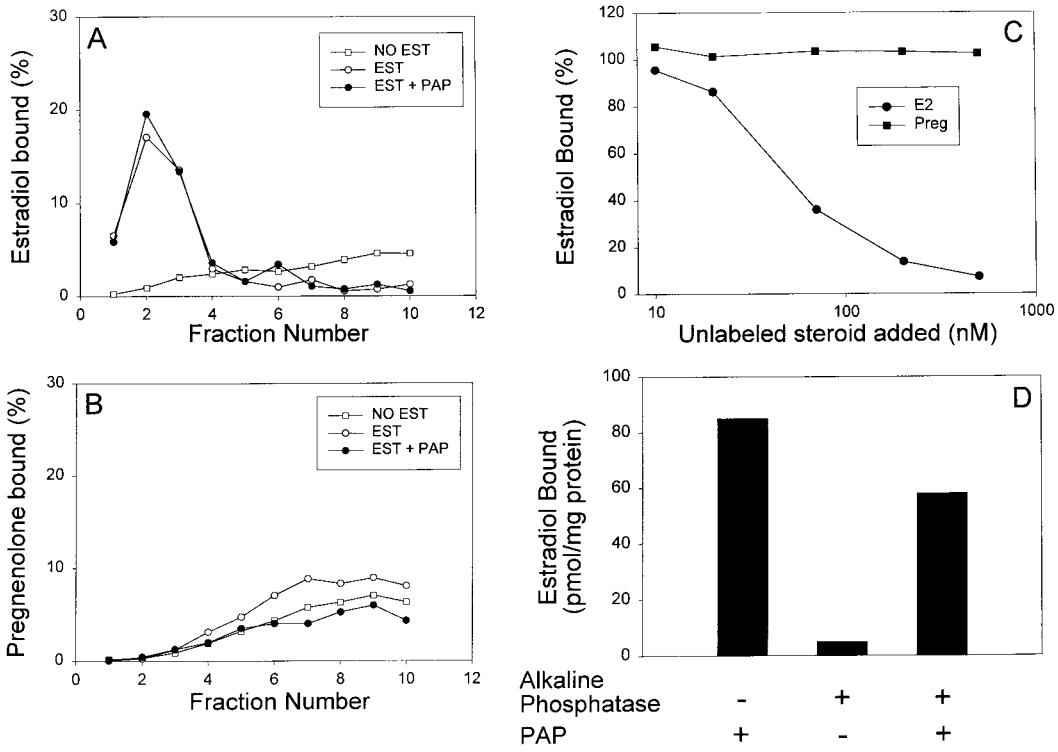


Fig. 1. Recombinant mouse EST binds estradiol but not pregnenolone in a PAP-dependent manner. (A) and (B) gel filtration assays of estradiol- (A) and pregnenolone- (B) binding activity. Steroids (1 nM of estradiol or 10 nM of pregnenolone) were incubated with 10 μg protein in 500 μl Tris–EDTA buffer either in the presence (●) or absence (○) of 100 μM exogenous PAP. Control incubations with PAP and steroids but without EST protein (□) were also carried out. Amount of protein bound steroid is expressed as a percentage of total radioactivity added to the incubation mixture. (C) binding of ^{3}H-labeled estradiol to recombinant mouse EST was competed by excessive amount of nonradioactive estradiol but not pregnenolone. Incubations were carried out with 100 μM PAP as in A and the first three column fractions (see A) were combined and total radioactivity measured. Result is expressed as a percentage of radioactivity measured when no unlabeled steroid was added (regarded as 100%). (D) Estrogen-binding activity of the mouse estrogen sulfotransferase requires PAP as a cofactor. Control (−) or alkaline phosphatase-treated (+) pure recombinant mouse EST (10 μg each) was incubated with 1 nM ^{3}H-labeled estradiol in 500 μl TE in the presence (+) or absence (−) of 100 μM PAP. Amount of protein bound estradiol was determined as in C.

ATG translation start codon of the human EST cDNA [27] and contains a *Sma*I restriction site (CCCGGG). The downstream primer corresponds to the end sequence in the coding region of the cDNA [22]. The amplified cDNA fragments were analyzed on agarose gels (1.2%), purified with the Promega Wizard PCR purification Kit (Promega Corp., Madison, WI) and cloned into the pCRII vector (TA cloning Kit, Invitrogen, San Diego, CA). After appropriate restriction digestion to confirm its identity [27], the cDNA was excised from pCRII with *Sma I* and *Not I* and ligated into the fusion protein expression vector pGEX-4T-1 at the same sites. Expression in bacteria and purification of human EST was carried out using the same protocol previously described for the production of the mouse enzyme [12].

2.4. Western blot and enzyme activity assays

To prepare cytosolic proteins, CHO cells were washed with phosphate buffered saline, scrapped off the plate and resuspended in 10 mM Tris–HCl, pH 7.5, containing 0.25 M sucrose, 1 mM DTT, 1 mM PMSF and 10% glycerol. Cells were broken up by sonication on ice. The cell lysate was cleared by centrifugation at 15,000 × *g* for 15 min and supernatant collected. Protein concentration was determined by the Bradford method using a colorimetric assay kit from Bio-Rad (Richmond, CA). For Western blot analysis, total cytosolic proteins were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels (20 μg per lane), transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH; BA85, 0.45 μm) and probed with a rabbit polyclonal antiserum previously developed for the mouse EST [12]. Immunodetection was made with the enhanced chemiluminescence Western blotting detection system from Amersham (Arlington Heights, IL).

Enzyme activity assays were carried out using ^{3}H-labeled estradiol, pregnenolone and dehydroepiandrosterone (25–87.6 Ci/mmol, DuPont, NEN, final concentration 1.25 nM) in 200 μl of 200 mM Tris–acetate pH 7.9 buffer containing 10 mM Mg–acetate, 1.25% Triton X-100, 100 μM PAPS (or with specified concen-

trations of PAPS) (Sigma, St. Louis, MO) and appropriate amount of either pure EST protein or total CHO cell proteins. Reactions were initiated by addition of the substrate and continued for 30 min at 37°C. At the end of the reaction, the mixture was extracted with 2 volumes of dichloromethane and aliquot of the aqueous phase was counted and taken as a measure for the amount of sulfated product [12].

2.5. Steroid-binding assays

Steroid binding activities were measured by incubating 10–30 μg pure EST protein or 200 μg CHO cell cytosolic proteins with specified concentrations of ^{3}H-labeled estradiol, dehydroepiandrosterone or pregnenolone and PAP (Sigma, St. Louis, MO) in 500 μl 20 mM Tris–HCl, pH 7.5 and 1 mM EDTA (TE buffer). The incubation was carried out on ice for 30 min and the mixture applied to a self-packed Sephadex G25 column (200 mg dry Sephadex G25 powder per column, pre-equilibrated with TE buffer) to separate EST-bound estradiol or pregnenolone from the free steroid. The flow-through fraction of the reaction mixture was collected as fraction 1 (0.5 ml). The column was then washed with TE buffer and 0.25 ml fractions were collected. Fractions were mixed with scintillation fluid and radioactivity determined. In some experiments, only the first three fractions were collected, combined and counted for EST-bound radioactivity. For competition studies, incubations were first carried out with nonradioactive steroids at the specified concentrations for 30 min before ^{3}H-labeled estradiol or pregnenolone was added and incubated. To determine the PAP dependency of the steroid-binding activity of the mouse and human EST, pure EST protein or total CHO cell proteins were treated with alkaline phosphatase (New England Bio Lab) at 37°C for 30 min (8 units alkaline phosphatase per 10 μg proteins). At the end of the reaction, the alkaline phosphatase was inactivated by adding MOPS (pH 7.0) buffer and DTT to a final concentration of 0.1 M and 50 mM respectively [23]. Steroid-binding activities of the treated proteins were subsequently assayed in the presence or absence of 100 μM PAP in the incubation mixture.

3. Results

3.1. Bacterially expressed mouse EST binds estradiol but not pregnenolone in a PAP-dependent manner

In view of the reported high affinity pregnenolone- and estrogen-binding activity of the guinea pig adrenal gland EST [23], we first tested the ability of the bacterially expressed mouse EST protein to bind these two steroids by gel filtration chromatography. We found that the mouse enzyme displayed high estradiol-binding activity but possessed no pregnenolone-binding activity (Fig. 1A and B). When the incubation mixture was applied to the column and the eluent fractionated, a significant amount of estradiol radioactivity was found to co-elute with the early protein fractions (Fig. 1A). No radioactivity was detected in these fractions when EST protein was omitted from the incubation (Fig. 1A). In contrast to estradiol, essentially no pregnenolone radioactivity was found to co-elute with EST protein (Fig. 1B). This difference was observed despite the fact that pregnenolone was used in the incubation mixture at 10 times the concentration of estradiol. Furthermore, addition of excessive amount of nonradioactive estradiol, but not pregnenolone, to the incubation mixture could effectively diminish the amount of ^{3}H-labeled estradiol associated with the EST protein (Fig. 1C). This result indicated again that the binding of estradiol to the mouse EST was specific.

Using the pure recombinant mouse EST, a saturation curve of the estradiol-binding activity was generated and Scatchard transformation of the data revealed a single binding site on the mouse EST protein with an estimated k_d of 31 nM. Thus, the mouse EST binds estradiol with relatively high affinity as did the guinea pig adrenal gland EST. The estradiol-binding activity of the guinea pig adrenal EST has been shown to require PAP as a cofactor [23,24]. However, data from Fig. 1A showed that the bacterially expressed mouse EST had intrinsic estradiol-binding activity in the absence of exogenously added PAP. Addition of 100 μM PAP to the incubation mixture did not significantly increase the binding activity (Fig. 1A). To determine if the estradiol-binding activity of the mouse EST was PAP-dependent, the enzyme was first treated with alkaline phosphatase before the binding assay was performed. Fig. 1D shows that pre-treatment of the mouse EST with alkaline phosphatase completely abolished its estrogen-binding activity. However, estradiol-binding activity in phosphatase-treated mouse EST could be reconstituted by adding exogenous PAP to the incubation mixture (Fig. 1D). These results demonstrate that, similar to the finding with the guinea pig adrenal EST [23], the estradiol-binding activity of the mouse EST is dependent on PAP as a cofactor. They also implied that the bacterially expressed mouse EST existed largely in a PAP bound form.

3.2. Steroid-binding activity of mouse EST expressed in CHO cells

The above result showing that the bacterially expressed mouse EST binds estradiol but not pregnenolone was in clear contrast with the reported dual

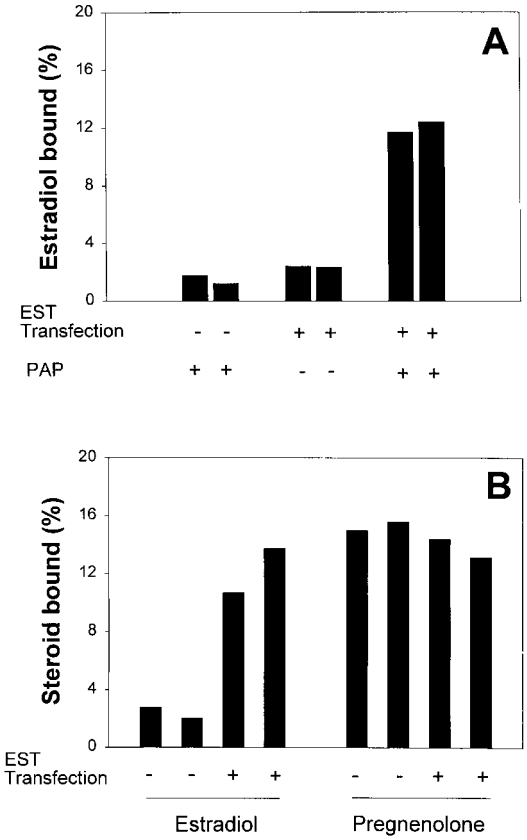


Fig. 2. Assay of estradiol- and pregnenolone-binding activity of recombinant mouse EST expressed in Chinese Hamster Ovary cells. (−): control CHO cells; (+): EST-transfected CHO cell. (A) EST transfection conferred CHO cells estradiol-binding activity which was dependent on exogenously added PAP. (B) In a second experiment, cellular proteins from the same culture dish were divided into two parts and were assayed separately for estradiol and pregnenolone-binding activity in the presence of 100 μM PAP. Two hundred micrograms of cytosolic CHO cell proteins were used in standard binding incubations (500 μl TE with 1 nM ^{3}H-labeled estradiol or 10 nM pregnenolone, with (+) or without (−) 100 μM PAP). Amount of protein bound estradiol or pregnenolone was determined as in Fig. 1C. Results from duplicate culture dishes are shown in each panel.

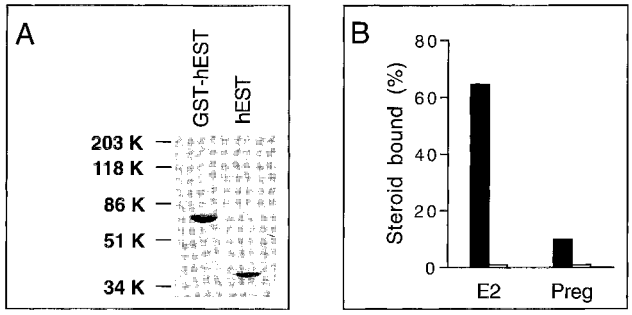


Fig. 3. Recombinant human EST binds pregnenolone as well as estradiol. (A) SDS-PAGE of purified bacterially expressed human EST (hEST) and GST-hEST fusion protein. Positions of molecular weight markers (in Da) are shown on the left. (B) Estradiol (E2) and pregnenolone (Preg)-binding activity of human EST. Result of a representative assay is shown. Filled bars: PAP added to the incubation mixture at 100 μM; Open bars: enzyme pre-treated with alkaline phosphatase and no exogenous PAP was added to the incubation. Incubation mixtures contained 20 μg human EST protein and 1 nM estradiol or 10 nM pregnenolone. Binding assays were carried out as described in Fig. 1.

binding activity of the guinea pig adrenal EST for estradiol and pregnenolone [23,24]. Since the results on the guinea pig adrenal EST were obtained from studies using EST protein expressed in CHO cells [23,24], we wondered if the pregnenolone-binding activity of EST might be dependent on certain post-translational modification(s) of the protein which was not occurring in bacteria but could be conferred by CHO cells. To test this possibility, we transfected CHO cells with the mouse EST cDNA using the eukaryotic expression vector pCDNA3. Fig. 2A shows that expression of mouse EST in CHO cells conferred estradiol-binding activity to these cells. It is of interest that, unlike the bacterially expressed protein, little of the CHO cell-expressed mouse EST appeared to have PAP bound and addition of exogenous PAP was necessary to demonstrate the estradiol-binding activity (Fig. 2A). Once again, in contrast to this clear estradiol-binding activity, no pregnenolone-binding activity could be detected in CHO cells expressing the mouse EST (Fig. 2B). As might be expected with the use of total cytosolic proteins, there were appreciable amount of non-specific protein bindings for the two steroids. Noticeably, the non-specific binding of pregnenolone was much higher than that of estradiol (Fig. 2B), presumably reflecting the more lipophilic nature of pregnenolone.

3.3. Recombinant human EST binds both pregnenolone and estradiol

The lack of pregnenolone-binding activity of mouse EST was in clear contrast with the guinea pig EST [23,24]. To further clarify this issue, we expressed and examined the potential pregnenolone-binding activity of the human EST protein. Using a GST-fusion protein expression procedure that we previously developed for the expression of the mouse enzyme [12], we successfully produced large amounts of pure and active human EST enzyme (>1 mg/l of bacteria culture) (Fig. 3A). Interestingly, binding experiments showed that recombinant human EST was able to bind both estradiol and pregnenolone, again in a PAP-dependent manner (Fig. 3B). Thus, it seems that the human EST resembles the guinea pig adrenal EST and not the mouse EST with regard to the pregnenolone-binding activity.

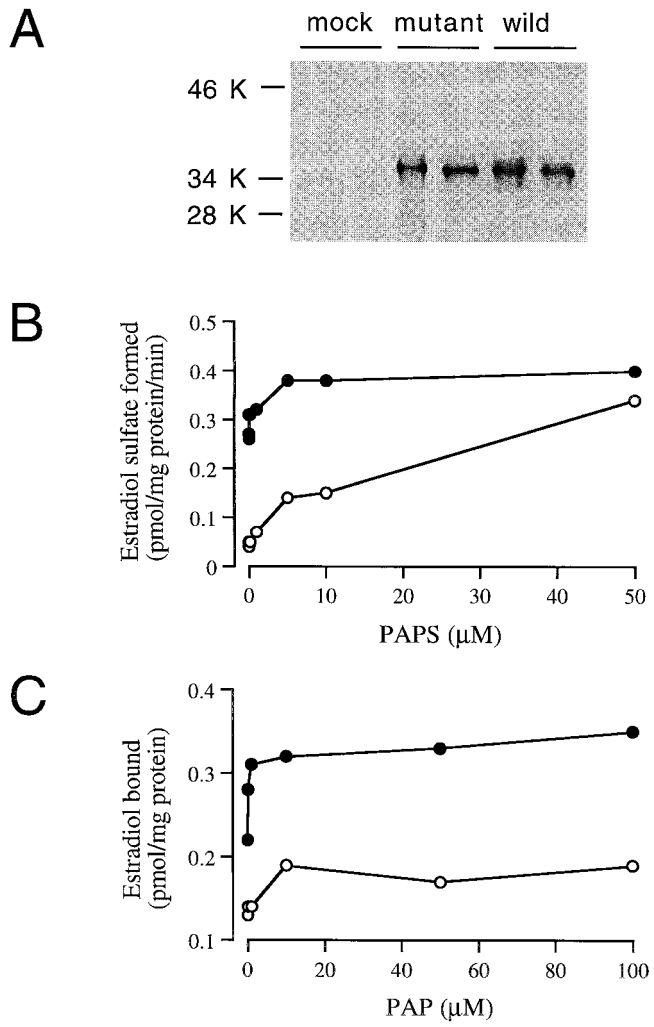


Fig. 4. Comparison of the estradiol-binding and sulfotransferase activities between the wild type and the G259A/G262A mutant mouse EST. (A) Western blot analysis of transfected CHO cells. Lanes 1 and 2, mock transfected cells; Lanes 3 and 4, G259A/G262A mutant EST transfected cells; Lanes 5 and 6, wild type EST transfected cells; 40 μg proteins were loaded in lanes 1, 3, 5 and 20 μg in lanes 2, 4, 6. Molecular weight standards (in Da) are shown on the left. (B) Sulfotransferase activities of CHO cell-expressed wild-type (●) and mutant (○) mouse EST (values represent average of triplicate assays). (C) Estradiol-binding activities of the wild-type (●) and mutant (○) proteins. Cytosolic proteins (200 μg) of transfected CHO cells were assayed with 1 nM ^{3}H-labeled estradiol and at the indicated concentrations of PAPS or PAP.

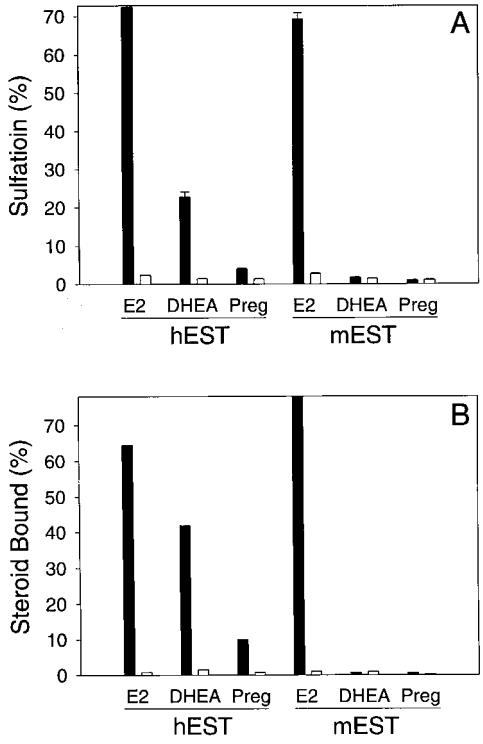


Fig. 5. Correlation between the specificity in steroid-binding and substrate specificity of the enzymatic activity of recombinant mouse and human estrogen sulfotransferases. (A) sulfotransferase activity presented as percentage of substrate conversion to the sulfated derivative (mean ± S.E.M., $n = 5$ independent determinations. Incubation volume: 200 μl; protein concentration: 0.1 μg; PAPS concentration: 100 μM; substrate concentration: 1.25 nM; incubation time: 30 min). Filled bars: native enzymes; Open bars: heat-inactivated enzymes. (B) steroid binding activities. Representative result of more than three independent binding assays is shown. Assays were carried out as described in the legend to Fig. 1 (amount of EST proteins: 10 μg; steroid concentration: 2 nM. Filled bars: PAP added to the incubation mixture at 100 μM; Open bars: enzyme pre-treated with alkaline phosphatase and no exogenous PAP was added to the incubation. E2, estradiol; DHEA, dehydroepiandrosterone; Preg, pregnenolone; hEST, recombinant human EST; mEST, recombinant mouse EST.

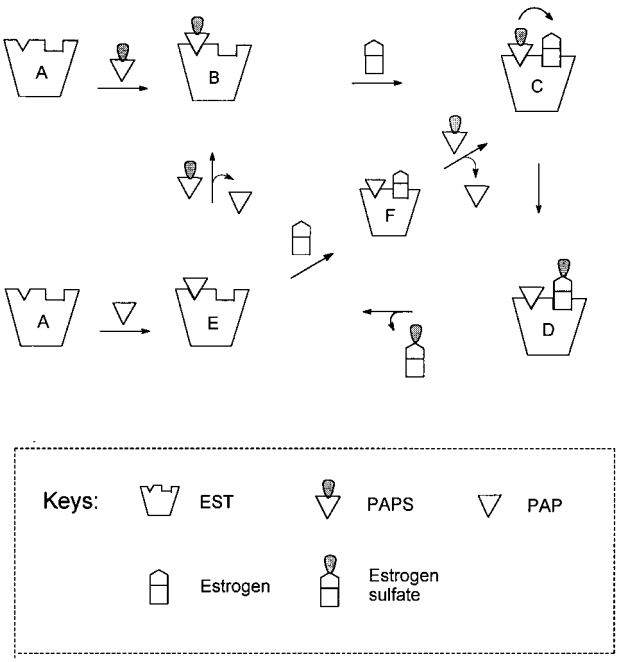
3.4. Site-directed mutagenesis within a domain critical for PAPS-binding affects both the estrogen-binding and sulfotransferase activities of the mouse EST

Given the obvious relationship between PAPS and PAP and the fact that all three estrogen sulfotransferases (guinea pig [23], mouse and human) had estrogen-binding activity in a PAP dependent manner, we considered it likely that the PAP-dependent estrogen-binding activity of EST is related to the catalytic mechanism of the enzyme. To explore this possibility, we tested the effect of mutating two critical glycine residues in a domain that has previously been determined to be critical for PAPS-binding and therefore estrogen sulfotransferase activity [12,25]. Fig. 4A is a Western blot analysis showing that both the wild-type mouse EST and the G259A/G262A double mutant could be efficiently expressed in CHO cells. As expected [25], the sulfotransferase catalytic efficiency of the G259A/G262A mouse EST mutant was significantly lower than that of the wild-type enzyme (Fig. 4B). At low concentrations of PAPS (< 5 μM), the mutated mouse EST was essentially devoid of sulfotransferase activity (Fig. 4B). However, increasing the PAPS concentration to over 50 μM restored most of the enzyme activity (Fig. 4B). Interestingly, the PAP-

dependent estradiol-binding activity was likewise dramatically reduced in the G259A/G262A mutant (Fig. 4C). These results suggest that PAPS and PAP are very likely to bind to the same site on the EST protein (Fig. 4C).

3.5. Correlation in the specificity between steroid-binding and sufotransferase activities

The mutagenesis data strongly suggest that the stable PAP-dependent estrogen-binding activity of EST is related to its catalytic activity. Based on this tentative conclusion, one would predict the relative specificity in steroid binding of mouse or human estrogen sulfotransferase to be reflected by its substrate specificity. Indeed, when recombinant human and mouse EST were compared for their substrate specificity, the human EST was shown to sulfate dehydroepiandrosterone and pregnenolone as well as estradiol, while the mouse EST only metabolized estradiol (Fig. 5) [12,28].



Scheme 1. Possible mechanism for the coupling between the sulfotransferase and the steroid-binding activities. The cofactor PAPS and steroid substrate (with estrogen as an example) bind to the enzyme in an orderly fashion (A → C). After transferring the sulfonyl group from PAPS to estrogen (C → D), the estrogen sulfate product is rapidly released from the enzyme but PAP remains on the cofactor-binding site (D → E). The PAP-bound enzyme is capable of binding another molecule of estrogen (E → F). Facile displacement of PAP by PAPS either before (E → B) or after estrogen-binding (F → C) regenerates the enzyme for a new catalytic cycle. In the absence of PAPS, stable PAP- and PAP/estrogen-bound complexes may be formed (A → E → F).

4. Discussion

As a specific metabolic enzyme, estrogen sulfotransferase may play an important role in modulating estrogen activity, both systemically as a hepatic enzyme and locally in estrogen-target tissues [1–9]. Not particularly compatible with this concept or well understood is an enigmatic pregnenolone and estrogen-binding activity described for the guinea pig EST [20–24]. The presence in the guinea pig adrenal cortex of a soluble protein capable of binding pregnenolone with high affinity was well recognized and supported by experimental evidence [20–24]. Since pregnenolone, formed from cholesterol by the Cytochrome P450 side chain cleavage enzyme (P450scc), is a common precursor for various steroidogenic pathways [29], the potential role for a pregnenolone-binding protein in the regulation of steroidogenesis can be easily envisioned [20–24]. Although pursued as a pregnenolone-binding protein, there is little doubt about the identity of the purified and cloned guinea pig adrenal estrogen sulfotransferase. The guinea pig EST has extensive sequence homology with other estrogen sulfotransferases [10,12,27,30] and COS-7 and CHO cells transfected with the cloned cDNA displayed estrogen sulfotransferase activity [11,23,31]. Most importantly, CHO cells transfected with the cloned guinea pig EST cDNA were shown to have pregnenolone-binding activity [24]. Thus, it was conclusive that the pregnenolone-binding activity of guinea pig adrenal cortex resides within the estrogen sulfotransferase enzyme [2,24].

The notion that EST, acting as a pregnenolone-binding protein, might play a role in regulating steroidogenesis is also compatible with its prominent expression in the placenta [10] and the testis [12–14], two other steroidogenic tissues. Thus, a central issue to be resolved in considering such a theory was whether the pregnenolone-binding activity is a common property shared by estrogen sulfotransferases from other sources. This question has been addressed in this study by using recombinant human and mouse estrogen sulfotransferases. We demonstrate conclusively that recombinant human but not mouse EST was able to bind pregnenolone in a PAP-dependent manner, establishing that pregnenolone-binding is not an universal property of all estrogen sulfotransferases. On the other hand, we show that both the human EST and the mouse EST share the high affinity and PAP-dependent estrogen-binding activity previously reported for the guinea pig adrenal EST [23,24].

With regard to the guinea pig EST, the relationship between the stable pregnenolone/estrogen-binding activity and the estrogen sulfotransferase activity has not been established [2,23,24]. The notion that pregnenolone-binding is a separate activity independent of the sulfotransferase activity came from results of earlier

studies which demonstrated that this activity could be separated from the major adrenal pregnenolone sulfotransferase activity [21,32,33]. Furthermore, in subsequent studies pregnenolone was found to compete with estradiol for the PAP-dependent binding to recombinant guinea pig EST but did not inhibit the estradiol sulfotransferase activity of the same protein [23,24].

Our data suggest that the PAP-dependent steroid-binding activity of estrogen sulfotransferase is coupled to the catalytic activity of the enzyme. This conclusion is supported by the fact that mutations of the mouse EST within a domain known to be critical for PAPS-binding affected both its sulfotransferase and estrogen-binding activities. Furthermore, a close correlation between the selectivity in steroid binding and the substrate specificity of the sulfotransferase activity was observed for both the human and mouse estrogen sulfotransferases. A model for the potential steps involved in the catalytic cycle of EST is depicted in Scheme 1 which could account for both activities. In this model, PAPS and PAP share the same binding site on the EST protein and the PAP-EST or PAP-EST-steroid (complexes E and F, Scheme 1) represents stable intermediates in the catalytic cycle of the sulfotransferase reaction. Facile displacement of PAP by PAPS in these intermediates readily regenerates the enzyme for a new cycle of catalysis. From this model, one would predict that the PAP bound forms of EST (complex E and F) to be catalytically active. Indeed, such a conclusion is supported by data from the guinea pig adrenal EST [22,23] and by our own results on the bacterially expressed mouse EST (Figs. 1A and 5). On the other hand, the PAP-EST-steroid complex (complex F) is not an obligatory intermediate in the sulfotransferase catalytic cycle (i.e. displacement of PAP by PAPS can occur before substrate binding takes place, from intermediate E to B rather than from E to F, Scheme 1). This is compatible with our mutagenesis data shown in Fig. 4. Mutation at G259 and G262 drastically reduced the maximum PAP-dependent estradiol-binding activity of the mouse EST (Fig. 4C). However, despite the apparently decreased PAPS affinity, the maximum estrogen sulfotransferase activity obtainable was not significantly affected (Fig. 4B).

An extrapolation of the above proposed scheme of EST catalysis is that depending on the relative concentrations of PAPS, PAP and estrogen in a given tissue, EST might exist in multiple cofactor and substrate-bound forms, a concept which is in agreement with the findings on the purified guinea pig adrenal cortex EST [22,23] and the bacterially expressed mouse EST (Fig. 1A). Whether such cofactor and/or substrate-bound forms of EST actually exist in tissues in vivo and if and how they may affect the biological activity of the enzyme remain to be established. The possibility has been raised previously that the function of EST might be subjected to regulation by PAP such that in the guinea pig adrenal cortex it functions more as an estrogen-binding protein than as an enzyme [2,23]. Results of the present study suggest that such a function of EST may be possible in other tissues and species as well.

In summary, we have shown in this study that pregnenolone-binding is not an universal property of all estrogen sulfotransferases. We have further established that the specificity in steroid-binding of estrogen sulfotransferases is correlated with the substrate specificity of their enzymatic activity. These results have clarified the relationship between the PAP-dependent pregnenolone-binding and estrogen sulfotranferase activities of EST and suggest that the physiological role(s) of EST is more likely to be related to estrogen inactivation (sulfation or binding) than regulation of steroidogenesis by acting as a specific pregnenolone-binding protein.

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