Pergamon

0960-0760(94)E0026-H

J. Steroid Biochem. Molec. Biol. Vol. 49, No. 2/3, pp. 161–165, 1994

0960-0760/94 $7.00 + 0.00

Estrogen Affinity Crosslinking to Tyrosinase-like Immunoreactive Proteins of Rat Uterine Nuclear Extracts

János Garai* and James H. Clark†

Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030, U.S.A.

We have previously described tyrosinase-like proteins of rat uterine nuclear extracts with type II estrogen binding characteristics. In this paper we have been able to affinity label these polypeptides with radio-iodinated estradiol. The major label at ~33–38 kDa comigrates with a ~36 kDa tyrosinase immunoreactive band assessed by autoradiograms and Western blots following electrophoresis. A minor label was also detected at ~45 kDa. The label is attenuated by excess quercetin hence these proteins are believed to represent putative type II estrogen binding sites that bind this bioflavonoid. These estrogen binding proteins are distinct from the estrogen receptor as judged by immunoblotting. The affinity crosslinking will be a useful approach in the purification of tyrosinase like proteins.

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INTRODUCTION

We previously reported estrogen binding associated monophenolase and diphenolase enzyme activities [1] as well as the presence of a tyrosinase like immunoreactivity [2] in rat uterine nuclear extracts. Since the enzyme activities and the estrogen binding are reduced or lost during chromatographic purification, we chose an alternate approach for identification of the protein(s) involved. The purpose of these experiments was to adapt a recently reported photoaffinity method [3] to label uterine nuclear monophenolase–diphenolase activity and examine its relationship to tyrosinase like immunoreactivity.

Monophenolase and diphenolase attack on the phenolic 'A' ring of estrogens is known to produce highly reactive quinones and semiquinones [4, 5] capable of crosslinking to any protein with available sulfhydryl groups [6]. Though this may seem to prevent rational use of an affinity labeling approach the intermediate(s) formed by tyrosinase tends to combine with functional groups of proteins immediately after its formation [6]. Therefore the bulk of the covalent crosslinks was expected to label proteins directly involved in the conversion of the monophenolic estrogens.

*On leave from the Department of Pathophysiology, University Medical School of Pécs, Szigeti u. 12. Pécs, Hungary 7643.
†Correspondence to J. H. Clark.
Received 25 Oct. 1993; accepted 21 Jan. 1994.

EXPERIMENTAL

Materials

[16α-^{125}I]iodo-estradiol (220 Ci/mmol) and EN^{3}HANCE fluorography amplifier were purchased from New England Nuclear (Boston, MA, U.S.A.). 6-(*O*-carboxymethyl)oximino-(2-[^{125}I])iodo-histamine -estradiol (2000 Ci/mmol), [2, 4, 6, 7-^{3}H]estrone (90 Ci/mmol), horseradish peroxidase (HRP) linked protein-A, and enhanced chemiluminescence (ECL) detection reagents were the products of Amersham (Arlington Heights, IL, U.S.A.). Sephadex LH-20 lipophylic resin was from Pharmacia (Uppsala, Sweden). [^{125}I]protein-A (0.97 mCi/ml) was from ICN (Costa Mesa, CA, U.S.A.). Acrylamide, bis-acrylamide, TEMED, and β-mercaptoethanol were from Bio-Rad (Richmond, CA, U.S.A.). Spheroidal hydroxylapatite was obtained from BDH Chemicals (Poole, England). The nitrocellulose membranes were "TM-NC4" from Hoefer Scientific Instruments (San Francisco, CA, U.S.A.) or "Hybond-ECL" from Amersham. Other enzymes and chemicals were of analytical grade from Sigma (St Louis, MO, U.S.A.) and Fisher (Pittsburgh, PA, U.S.A.).

The lyophilized anti-tyrosinase immunoglobulins [7] (sample "H5" and "H7") raised against hamster melanoma enzyme [8] in rabbits were generous gifts from Dr Seymour H. Pomerantz (University of Maryland, Baltimore). Mouse monoclonal antibody "H_{151}", that was raised against a sequence in the hinge region

of the human estrogen receptor (Edwards, Tullos, Beck and Weigel; manuscript in preparation), and human estrogen receptor (hER) expressed in yeast [9], were kindly provided by Dr Nancy L. Weigel (Baylor College of Medicine).

Animals and tissue preparation

Female Sprague–Dawley rats 21 days old were obtained from Sasco Inc. and were implanted the next day after shipment with 100 μg estradiol-17β in s.c. beeswax pellets under short methoxyflurane anaesthesia (Metofane from Pitman Moore, Mundelein, IL, U.S.A.). The animals were sacrificed 71–73 h following the implantation. Uteri were removed and the tissue processed as described in previous papers [2, 10], with a more stringent nuclease treatment that was found to facilitate extraction of nuclear type II estradiol binding sites. Briefly, washed crude nuclear pellets were digested with DNase I (DN-25; 200 μg/ml) RNAse A (Type IIIA; 40 μg/ml), and RNase T_1 (20 U/ml) in buffer-B (110 mM NaCl, 10 mM Tris, 1.5 mM $MgCl_2$; pH 7.4) at 30°C for 25 min. Subsequent steps were carried out according to the original description [2, 10]. The extracted proteins were precipitated adjusting $(NH_4)_2SO_4$ concentration to 40% and stored at −70°C. The proteins were reconstituted in T-NP buffer (10 mM Tris; 0.1% NP-40, pH 7.4) 30–60 min prior to use. Protein content of the samples was assessed by the Bio-Rad Bradford assay [11] using bovine serum albumin as standard.

B16 melanoma cell extract was prepared by exposure of the harvested cells to 1% Nonidet P-40 plus 0.01% sodium dodecyl sulfate in 20 mM potassium phosphate buffer (pH 7.2) for 1 h at 4°C. Short bursts of sonication were applied four times along the incubation period to aid solubilization. The particulate material was pelleted by 20,000 *g* to obtain the supernatant containing solubilized tyrosinase.

Affinity crosslinking

For the crosslinking procedure the method of Poirot *et al.* [3] has been modified as follows. Briefly, reconstituted proteins of the uterine nuclear extract were adsorbed onto spheroidal hydroxylapatite suspension (HAP; 60% v/v in 10 mM Tris, pH 7.4) then washed with 10 mM Tris buffer to remove unbound proteins. The samples were incubated with the radioligands for 60 min at 30°C in Tris medium containing 10 μM $CuCl_2$ and 20 μM ascorbic acid. These conditions were found optimal for uterine nuclear tyrosinase like activity previously [1]. Where indicated UV irradiation was applied by a "Mineralight" model UVG-11 lamp through a glass plate throughout the incubation period. After the HAP suspension was washed in an excess volume of Tris buffer for removal of unbound radioglands it was packed into a minicolumn and proteins eluted with 0.4 M potassium phosphate + 0.1 M $(NH_4)_2SO_4$. The eluted proteins were applied to Sephadex LH-20 lipophylic resin minicolumns to adsorb remnants of non-covalently bound ligand. Eluted proteins of LH-20 were precipitated with 5% trichloroacetic acid, washed twice with ethanol–ether (2:1) and dried.

SDS–polyacrylamide gel electrophoresis, Western immunoblotting, and autoradiography

The proteins were separated on 10% polyacrylamide gel according to the method of Laemmli [12] with 0.1% SDS using bromophenol-blue as the tracking dye. Gels were loaded with 30 μg protein per lane. The prestained molecular weight markers of Amersham were: lysozyme (14.3 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (30 kDa), ovalbumin (46 kDa), bovine serum albumin (BSA, 69 kDa), phosphorylase *b* (97.4 kDa), and myosin (200 kDa). Gels were run in Mighty-Small II (Hoefer) chambers (25 mA/gel).

The separated proteins were transferred onto nitrocellulose membranes and Western blots were processed using milk protein "blotto" and [^{125}I]protein-A detection as described previously [2]. Immunoblotting for ER was done as described previously by Edwards *et al.* Briefly, the gels were equilibrated for 3 × 20 min with the transfer buffer (50 mM sodium phosphate; 0.05% SDS, 20% methanol, pH 6.8) prior to overnight transfer at 150 mA, 4°C. The membranes were blocked for 2 h at room temperature with 3% BSA in NEHTG buffer (50 mM HEPES; 5 mM EDTA 150 mM NaCl; 0.05% Triton-X100; 0.25% gelatin, pH 7.4), placed for 3 h in a solution of 4 M urea + 150 mM NaCl + 20 mM EDTA-pH 7.0; then washed in NEHTG buffer for 3 × 5 min. The membranes were incubated overnight at 4°C with hER antibody (1:1000) in NEHTG buffer containing 1% BSA, then, after three washes, with the secondary antibody (1:5000) for 4 h at room temperature. After four washes the membranes were reacted with HRP-linked protein-A (1:10,000) for 1 h at room temperature, washed, and the ECL image developed according to the manufacturer's instructions. When using ECL detection the latter procedure was applied also to the tyrosinase immunoblots (1:400 for H5; 1:2000 for H7) with omission of the secondary antibody.

To detect [^{3}H]estrone labeled proteins the nitrocellulose membrane was sprayed with ENHANCE and the fluorogram was obtained with 56 days exposure at −78°C. Autoradiograms of [^{125}I]estradiol labeled proteins were obtained with 32 days exposure.

RESULTS

[^{3}H]estrone is primarily localized at the ~60–65 kDa position in the uterine nuclear extracts. A minor label also migrates in the ~45 kDa range (Fig. 1). Uterine cytosol was characterized by a weakly labeled band at ~45 kDa (not shown). Since the crosslinking

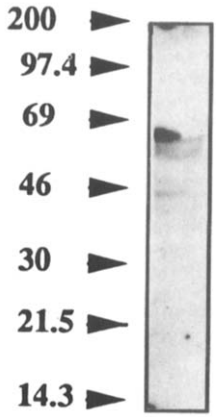

Fig. 1. [^{3}H]estrone labeled proteins of uterine nuclear extract. Uterine nuclear extract (0.6 mg protein/initial sample) adsorbed onto HAP suspension, was incubated for 1 h at 30°C with 70 nM [^{3}H]estrone while UV irradiated. Subsequent steps of sample preparation and fluorography were performed as described in "Experimental". Arrowheads indicate positions of the molecular weight markers (kDa).

efficiency is low we can only infer that such linkages have occurred. For better detection, ^{125}I ligands were substituted for [^{3}H]estrone in an attempt to correlate estrogen-crosslinkable proteins with tyrosinase immunoreactivity.

Either [16 α-^{125}I]estradiol or [^{125}I]histamine-tagged estradiol labels a ~33–38 kDa band that apparently comigrates with a ~36 kDa tyrosinase-immunoreactive polypeptide (Figs 2 and 3). The membranes were split after transfer hence the Western blots and the ^{125}I

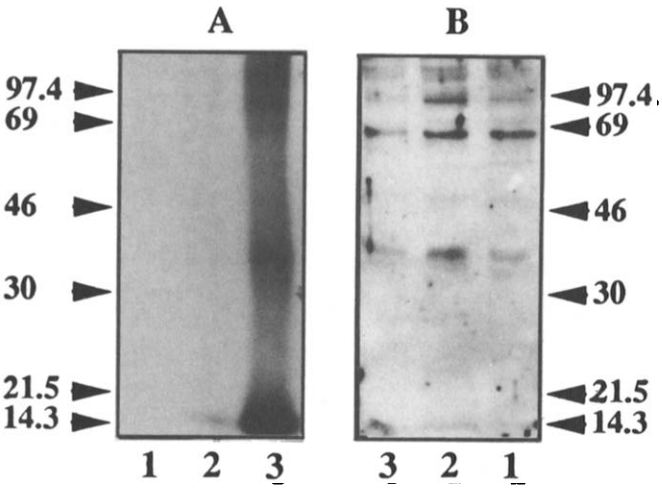

Fig. 2. [16α-^{125}I]estradiol labeled proteins and tyrosinase immunoreactivity of the uterine nuclear extract. Uterine nuclear extract (0.4 mg protein/initial sample) adsorbed onto HAP suspension, was incubated for 1 h at 30°C with 16 nM [16α-^{125}I]estradiol in the presence of a 600-fold excess of quercetin (lane 1), or diethylstilbestrol (lane 2), or in the absence of competitor (lane 3). Subsequent steps of sample preparation were performed as described in "Experimental". Autoradiograms (A) and tyrosinase immunoblots by the ECL method (B) were derived from the same nitrocellulose membrane split after protein transfer. Arrowheads indicate positions of the molecular weight markers (kDa).

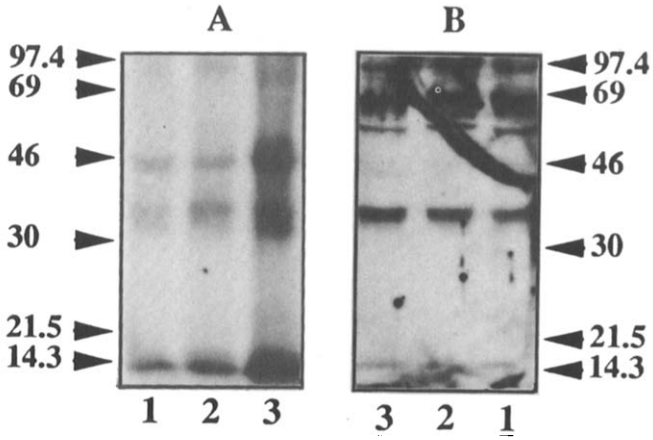

Fig. 3. ^{125}I histamine estradiol labeled proteins and tyrosinase immunoreactivity of the uterine nuclear extract. Uterine nuclear extract (0.6 mg protein/initial sample) adsorbed onto HAP suspension, was incubated for 1 h at 30°C with 8 nM [^{125}I]histamine estradiol in the presence of a 600-fold excess of quercetin (lane 1), or diethylstilbestrol (lane 2), or in the absence of competitor (lane 3) while UV irradiated. Subsequent steps of sample preparation were performed as described in "Experimental". Autoradiograms (A) and tyrosinase immunoblots by the ECL method (B) were derived from the same nitrocellulose membrane split after protein transfer. Arrowheads indicate positions of the molecular weight markers (kDa).

autoradiograms were derived from the same gels. With [^{125}I]histamine estradiol an additional band is labeled at ~45 kDa that can also be correlated with a weakly immunoreactive band. A considerable amount of label was incorporated into polypeptides migrating at the front. The labeling was attenuated either by excess diethylstilbestrol or quercetin. Although blunt label can be detected at higher molecular weight regions, discernible [^{125}I]estradiol crosslinks were not obtained at the position of the major ~68 kDa immunoreactive band.

To validate our immunoblots the previously used [^{125}I]protein-A detection was compared to the ECL method. These techniques yield comparable results in recognizing both the uterine tyrosinase like immunoreactive protein and the tyrosinase extracted from B16 murine melanoma cells [Fig. 4(A and B)]. In our experience lower molecular weight tyrosinase immunoreactive bands of the uterine nuclear extracts appeared with consistency after incubation at 30°C, e.g. during the crosslinking procedure and less frequently in extracts not exposed to that treatment. Nevertheless, this does not seem peculiar to the uterine tyrosinase-like protein, since a similar banding pattern of the B16 melanoma tyrosinase emerged in the crude melanoma cell extract upon 60 min preincubation at 30°C [Fig. 4(C)].

The estrogen receptor is abundant in the uterus, therefore the possible contribution of its estrogen binding function to the results presented here needed to be addressed. Using the monoclonal antibody "H_{151}"

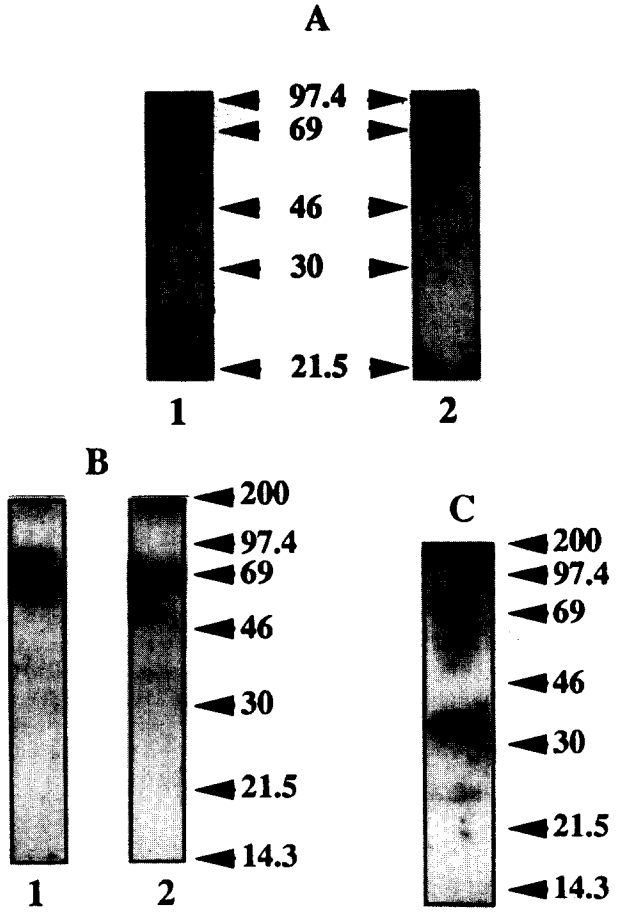

Fig. 4. Tyrosinase immunoreactivity of B16 murine melanoma cell extract in comparison with the uterine nuclear extract. (A) Tyrosinase immunoblot of uterine nuclear extract (lane 1) and B16 melanoma cell extract (lane 2) obtained with [^{125}I] protein A detection. (B) Tyrosinase immunoblot of B16 melanoma cell extract (lane 1) and uterine nuclear extract (lane 2) obtained with ECL detection. (C) Tyrosinase immunoblot of B16 melanoma cell extract with [^{125}I] protein A detection following 1 h preincubation at 30°C. Arrowheads indicate positions of the molecular weight markers (kDa).

developed against human estrogen receptor, immunoreactivity was not detected in the nuclear extract while ER was present in the uterine cytosol (Fig. 5).

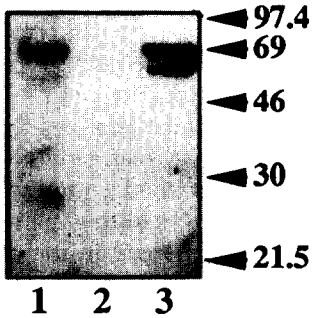

Fig. 5. Estrogen receptor immunoreactivity of the uterine nuclear extracts. ER immunoblot of uterine cytosol (lane 1), the uterine nuclear extract (lane 2) and of human ER expressed in yeast (lane 3) using ECL detection. Arrowheads indicate positions of the molecular weight markers (kDa).

DISCUSSION

The consistent comigration of estrogen crosslinked bands with tyrosinase immunoreactive bands of Western immunoblots derived from the same gel indicates that these are likely to represent identical protein(s). The appearance of the lower molecular weight tyrosinase immunoreactive bands might be the result of either subunit dissociation or proteolysis of the major ~68 kDa moiety. The label found in the front on autoradiograms may as well be attributable to small proteolytic fragments of the estrogen crosslinked protein(s). Production of the lower molecular weight form(s) of the tyrosinase like protein might be a prerequisite for and/or a consequence of the estrogen binding. Further studies are required to substantiate the validity of these premises.

The differences in the pattern of crosslinked bands obtained with the different estrogen radioligands used here are probably due to the following factors: estrone features the 17-keto group that is considered responsible for its photoreactivity. The ^{125}I labeled estradiol compounds do not have this group hence are not expected to be highly reactive on light irradiation. UV irradiation of the [16α-^{125}I]estradiol is in fact not recommended by the manufacturer due to its photosensitivity, therefore it was not attempted. Its lower specific activity, in addition, also renders detectability of the crosslinks formed by [16α-^{125}I]estradiol [Fig. 2(A)] inferior compared to the result obtained with [^{125}I] histamine estradiol [Fig. 3(A)].

Based on the above considerations a mechanistic scenario can be proposed. The highest molecular weight (~60–65 kDa) affinity labeled band detected exclusively with [^{3}H]estrone and UV irradiation represents a crosslink between the protein and the photoreactive 17-keto group that is presumably formed instantly upon binding of this radioligand and partially prevents the interrelated further processing of probably both the protein and the ligand. On the other hand, due to a ligand induced activation of the enzyme catechols and quinones are produced from the radio-iodinated estradiols lacking the 17 keto group and these are capable of crosslink formation with the protein as it is processed into smaller molecular weight forms. These ligands appear to be incorporated mainly into the ~33–38 and, to some extent, into the ~45 kDa bands on the autoradiograms. Photooxidation of the catechols and quinones into more reactive semiquinones [13] may in fact promote instant crosslinking that might be responsible for a more prominent labeling of the ~45 kDa band upon UV irradiation.

Although the outcome here may appear similar to the "meroreceptor" concept described for the steroid receptors [14] the uterine nuclear extracts derived by the particular extraction procedure used throughout this study do not contain the classic estrogen receptor as judged by immunoblotting. The estrogen affinity

labeled protein(s) of these uterine nuclear extracts clearly differs from the estrogen receptor.

The bioflavonoid quercetin, a potent ligand of type II estrogen binding sites [15], and the synthetic estrogen diethylstilbestrol both interfered with incorporation of the label. We propose that the ~33–38 kDa band represents estrogen crosslinked to the 37 kDa form of the uterine nuclear type II estrogen binding site [10], while the ~45 kDa band may be identical to a variant form labeled previously in rat uterine cytosol [3]. Both of these forms are likely to be derived from the major ~68 kDa tyrosinase immunoreactive protein in a process that may bear significance to the action and/or metabolism of estrogens in the uterus.

The origin of a tyrosinase immunoreactivity in non-pigmented tissues is obscure at present, since in mammals authentic tyrosinase and tyrosinase related proteins are known to be expressed in a tissue specific manner only in neural crest derived melanocytes [16]. Moreover, albino rats have been used in this study. However, the domain(s) of the uterine protein responsible for the immunoreactivity are expected to share some structural and probably also functional features with the authentic tyrosinase. Purification and sequence information of the tyrosinase immunoreactive estrogen binding protein is needed to cast light on these issues.

Acknowledgements—This work was supported by NIH Grant HD 08436-19. We wish to thank Dr Seymour H. Pomerantz (Department of Biochemistry, School of Medicine, University of Maryland at Baltimore) for the tyrosinase antibodies. We would also like to thank Dr Nancy L. Weigel for the hER and for the antibodies for the ER immunoblot and Dr Kathy Jackson for providing the melanoma cells.

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