IDENTIFICATION OF THE 'ESTROGEN-INDUCED PROTEIN' IN UTERUS AND BRAIN OF UNTREATED IMMATURE RATS

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1. Introduction

The appearance of a specific estrogen-induced protein (IP) in the rat uterus [1] represents one of the earliest effects of estrogen on uterine macromolecular synthesis [2]. This effect can be reproducibly demonstrated following in vitro as well as in vivo estrogen treatment [3–5], making IP the most convenient marker protein available for studying the mechanism of action of estrogen in the rat uterus.

IP has been routinely detected using a double-isotope ratio method [6]. This method is convenient, but it cannot yield information on content or rate of synthesis of IP in non-treated organs, or in organs in which IP may be present but not susceptible to induction by estrogen.

We have reported initial results, on the time course and age dependence of IP induction [7], using fluorography of [^{35}S]methionine-labelled proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis [8]. We describe here the characterization of IP by partial protease digestion, which enables the identification of IP as a major constitutive protein in uterus, pituitary, hypothalamus and cerebral cortex of immature rats. (A short report of some of these findings was presented [9].)

2. Materials and methods

2.1. *Biochemicals*

[^{35}S]Methionine (250–400 Ci/mmol) was obtained from the Radiochemical Centre Amersham. Estradiol-17β was purchased from Ikapharm Inc., Ramat-Gan; 'Cellogel', gelatinised cellulose acetate was from Chemetron, Milan, and Coomassie brilliant blue from Schwartz/Mann, Orangeburg, NY. *Staphylococcus aureus* protease was purchased from Miles, Slough.

2.2. *Preparation and fractionation of cytosol samples*

Immature female rats from the departmental colony (25 days) were the source of all organs unless otherwise stated. Rats were injected intraperitoneally with 5 μg estradiol-17β in 0.5 ml 1% ethanol or with 1% ethanol vehicle. Rats were killed 1 h after injection, organs were excised, rinsed in phosphate buffered saline (PBS) [10] and incubated for 2 h at 37°C, under a 95% O_2/5% CO_2 atmosphere, in PBS containing 50 μCi [^{35}S]methionine. Organs were homogenised in an all-glass Potter-Elvejehm homogeniser (0.005–0.007 in. clearance) in 5 vol. 10 mM Tris–HCl (pH 7.5), 1 mM EDTA, and the homogenate centrifuged at 2°C for 50 min at 198 000 × g. For partial purification of IP from cytosol, electrophoresis on preparative Cellogel blocks (2.5 mm and 5 mm thick) was used [11]. The material having a mobility of 1.05–1.20 relative to BSA (found to include IP) was recovered from the gel.

2.3. *Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis*

This was performed on 10–20% polyacrylamide gels according to [12]. Calibration curves for molecular weight were constructed according to the observed mobilities of bovine serum albumin (BSA), ovalbumin, hemoglobin and cytochrome *c*. For the purpose of fluorography, gels were dried, impregnated with a scintillator [13] and exposed to Kodak RP 54 X-ray film.

For digestion of protein samples with *S. aureus* protease the method in [14] was used: IP bands were cut from polyacrylamide slab gels. The gel slices were inserted into sample wells of a second polyacrylamide gel together with a solution containing 100 ng protease. Digestion was allowed to proceed in the stacking gel.

3. Results

3.1. *Identification of IP by sequential separation according to charge (cellogel) and size (SDS–polyacrylamide gel electrophoresis)*

Double isotope ratio analysis of amino acid-labelled cytosol has revealed that IP migrates during electrophoresis on Cellogel with a mobility of 1.1-times that of BSA [11]. Radioactive proteins of uterine cytosol were subjected to cellogel electrophoresis and the fractions obtained electrophoresed on SDS–polyacrylamide gels (fig.1). IP has been shown to be a mol. wt 46 000 protein of acidic nature (shown by its rapid migration on cellogel electrophoresis) and whose rate of synthesis is stimulated within 1 h of estrogen administration [15]. These properties are consistent with an identification of the band of M_r 46 000 from cellogel fraction 7 (corresponding to proteins with a mobility of 1.05–1.2-times that of BSA) as that containing IP (fig.1). IP was found almost exclusively in fraction 7

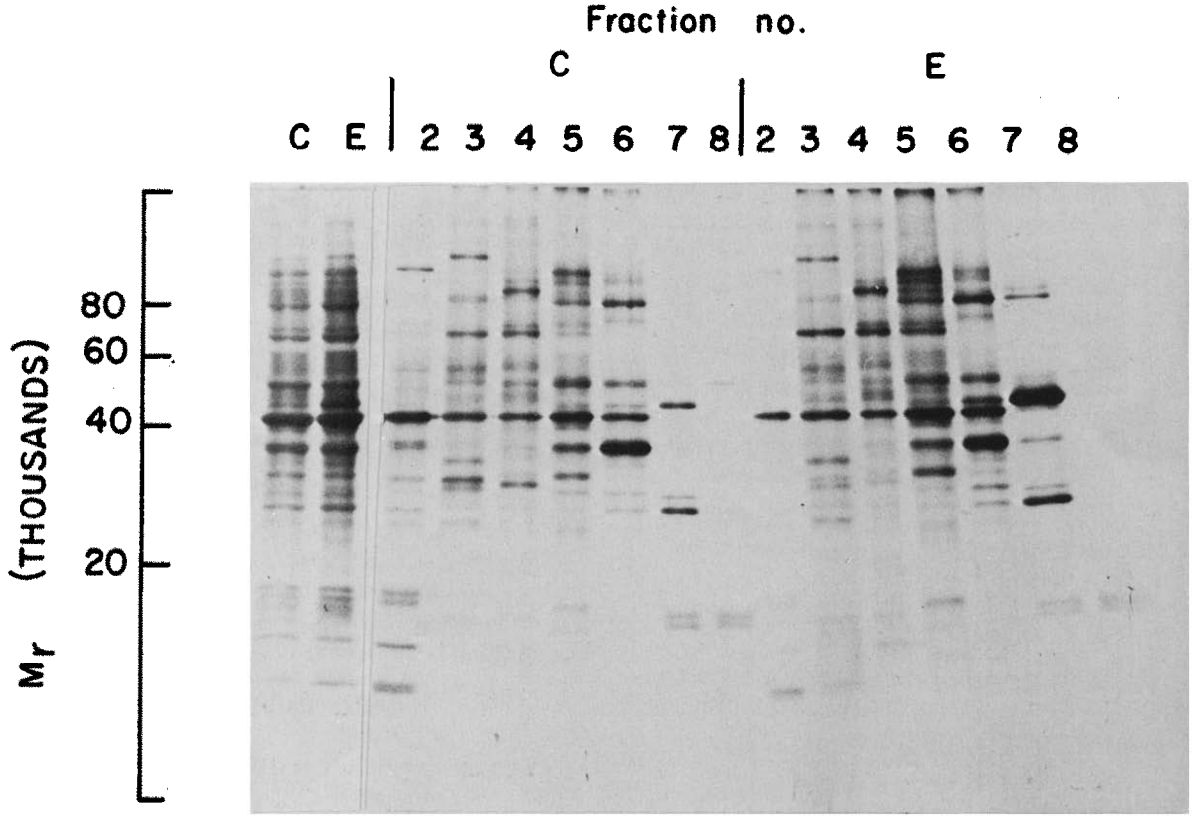

Fig.1. Fluorogram of SDS–polyacrylamide gel electrophoresis of unfractionated and cellogel-fractionated cytosol. Samples of ^{35}S-labelled uterine cytosol proteins from control (C) and estrogen-treated (E) rats were subjected to cellogel electrophoresis and separated into 8 fractions according to mobility relative to bovine serum albumin (BSA). Fraction: 1, –0.1 to +0.1 times mobility of BSA; 2, 0.1–0.3; 3, 0.3–0.5; 4, 0.5–0.7; 5, 0.7–0.9; 6, 0.9–1.05; 7, 1.05–1.2; 8, 1.2–1.4. Unfractionated cytosol and fractions 2–8 were then subjected to electrophoresis on a 10–20% polyacrylamide gel. The exposure time of the total cytosol samples was less than that of the fractions, in order to avoid over-exposure.

both in preparations derived from untreated and estrogen-treated animals.

3.2. *The presence of IP in rat brain and pituitary gland*

An SDS–polyacrylamide gel electropherogram of unfractionated cytosol and cellogel-fractionated cytosol from a number of rat organs is presented in fig.2. The cellogel fraction tested is that corresponding to the mobility of IP (fraction 7). In the case of cerebral cortex, hypothalamus and pituitary from female rats, as well as cerebral cortex of immature male rats, an IP-like protein was detected both in cytosol and cellogel-purified fractions. In the case of liver and muscle, no such clear band corresponding to IP was visible in unfractionated cytosol samples. The cellogel-purified fractions derived from muscle and liver cytosol were concentrated 5-fold relative to those of other rat organs. These concentrated fractions showed a faint band corresponding to IP, indicating a much lower IP content in muscle and liver than the other rat organs tested. Comparing the incorporation of [^{35}S]methionine into the IP-like protein of pituitary and hypothalamus, up to 2 h after estrogen administration, revealed no evidence for induction of this protein (data not shown). Because of relatively poor incorporation of [^{35}S]methionine into protein in vitro by cerebral cortex, possible IP-induction in this tissue has not yet been investigated.

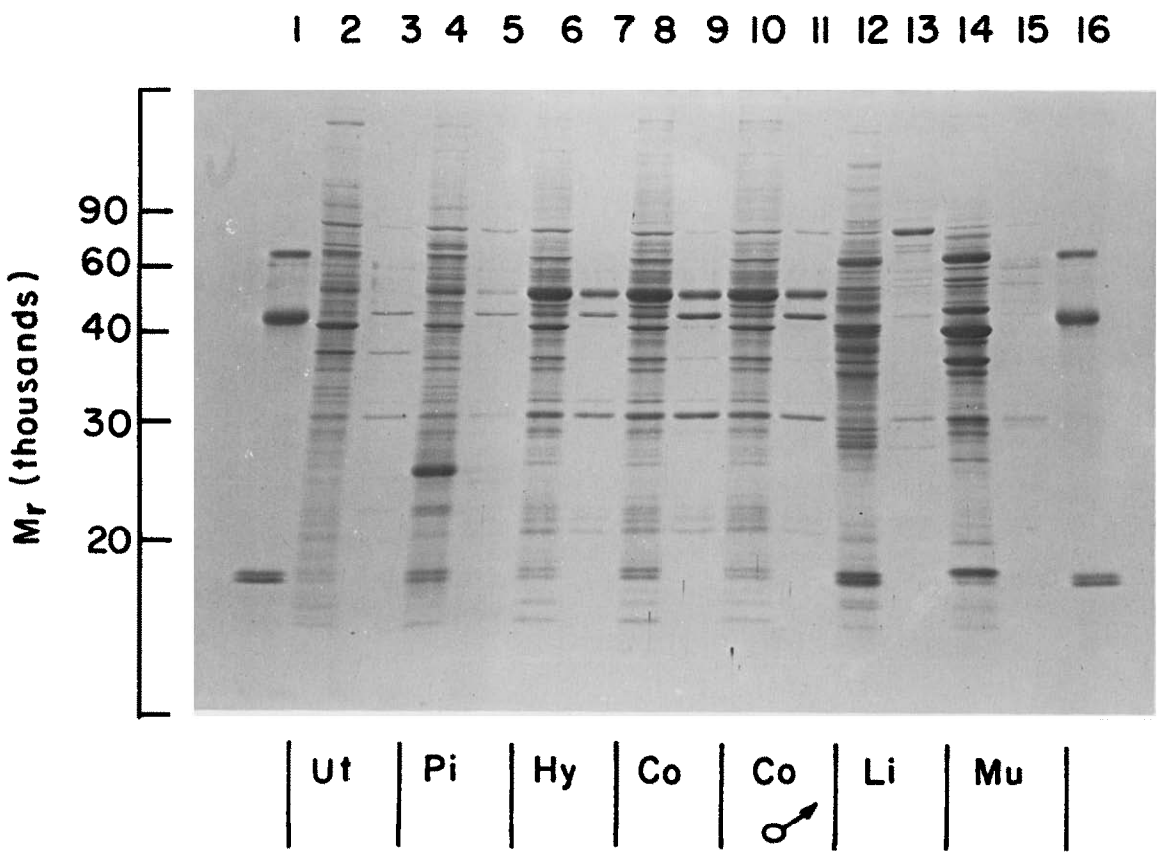

Fig.2. Coomassie brilliant blue staining pattern of SDS–polyacrylamide gel electropherograms of unfractionated cytosol and cellogel fractionated cytosol of uterus (Ut), pituitary (Pi), hypothalamus (Hy), cerebral cortex (Co), cerebral cortex of immature male (Co ♂), liver (Li) and muscle (Mu). The cellogel fraction shown is that corresponding to the mobility of IP (fraction 7 as shown in fig.1). Lanes 1 and 16 show molecular weight markers (BSA, 67 000; ovalbumin, 45 000; hemoglobin, 16 000). IP migrates slightly slower than ovalbumin.

3.3. *Comparison of protease partial digestion products of IP from several tissues*

In order to examine further the relationship between the various IP-like proteins and uterine IP, small amounts of these proteins were purified through the stages of cellogel and SDS–polyacrylamide gel electrophoresis, and subjected to partial protease digestion using the *S. aureus* protease V8. The digestion patterns of IP-like proteins from cerebral cortex, hypothalamus, pituitary and untreated uteri compared with uterine IP are shown in fig.3. The patterns of all materials are identical, implying a close similarity in the original proteins. A number of proteins of roughly similar molecular weight to IP were also digested. The digestion patterns of rabbit muscle actin and rat brain tubulin were clearly different from that of IP (fig.3).

4. Discussion

The limitations of double-isotope ratio analysis led us to search for improved methods of detecting IP. Use of SDS–polyacrylamide slab gel electrophoresis

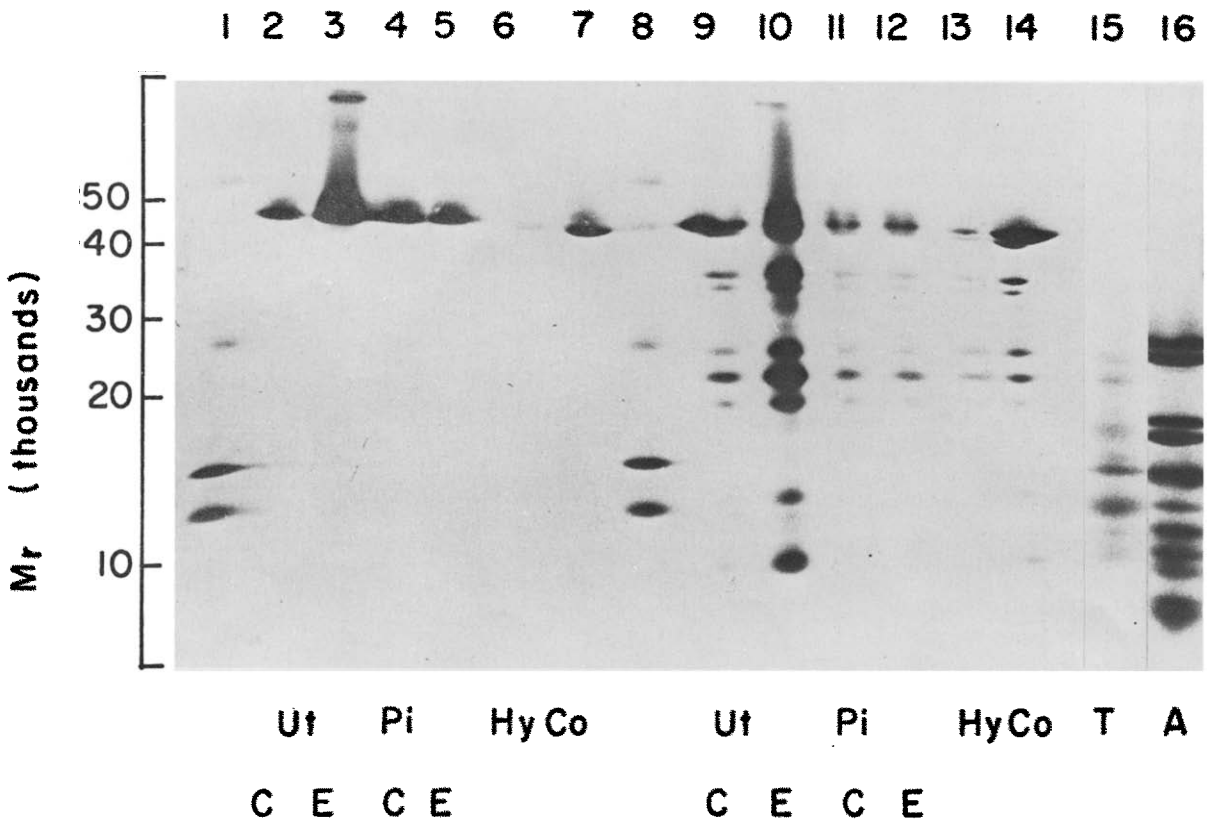

Fig.3. Protease partial digestion pattern of IP-like proteins from uterus (Ut), pituitary (Pi), hypothalamus (Hy) and cerebral cortex (Co). Purified samples of IP-like proteins from the above organs were prepared as in section 2, cut from SDS–polyacrylamide slab gels and subjected to electrophoresis on SDS 15–20% polyacrylamide gels in the presence or absence of *S. aureus* protease. Lanes 1 and 8 contain molecular weight markers (BSA, ovalbumin, α-chymotrypsinogen, myoglobin and cytochrome *c*). Lane 2–7: IP-like proteins in the absence of protease. Lanes 9–14: digestion pattern resulting from addition of protease. Organ abbreviations are those used in fig.2 legend. The letters under the organ abbreviations indicate preparations from control (C) and estrogen-treated (E) rats. Lanes 15 and 16 show rat tubulin (T) and rabbit muscle actin (A), respectively, in the presence of protease. The patterns for uterus and pituitary represent fluorograms of the gel after electrophoresis, whereas those for the remainder are Coomassie brilliant blue-staining patterns.

coupled with fluorography allowed us to unequivocally identify a particular protein band (at mol. wt 46 000) as the one which contains IP.

A protein showing the same molecular weight and electrophoretic mobility as IP was found to be present in cytosol and cellogel-purified fractions from cerebral cortex, hypothalamus, pituitary and untreated immature uteri. Identity of these IP-like proteins with IP from estrogen-treated uteri is strongly suggested by an exact correspondence of the pattern of peptides from a partial protease digest of these proteins. Brain may thus be a convenient source of material for large scale preparations of purified IP which are needed for studies of its structure and function. A parallel case, in which a progesterone-stimulated protein originally thought to be confined to the female reproductive tract has been found in the male reproductive tract and in the respiratory tract, is the work in [16,17] on rabbit uteroglobin.

IP has been reported [18] to act as a stimulant of DNA synthesis for 3T6 cells in culture. We have shown an age-dependent stimulation of DNA polymerase α in immature rat uterus following estradiol treatment [19]. This effect coincides with the observed age-dependent acquisition of responsiveness to estradiol measured by an increased rate of DNA synthesis [20] and follows closely attainment, during postnatal development, of the maximal level of stimulation of IP synthesis [21].

One hypothesis consistent with the above data is that there may be present in non-responsive cells constitutive levels of IP, sufficient to allow a maximal rate of growth: responsive cells, on the other hand, may require induction of IP by estrogen to attain levels necessary for maximal cell growth and proliferation. Measurements of the amount of IP in various cell types using radioimmunoassay techniques [22] should allow evaluation of this hypothesis.

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