EXPERIMENTAL STUDY

The nuclear, 75 kDa form of early growth response protein-1/nerve growth factor-induced A protein is primarily restricted to LH β -subunit-expressing cells in rat anterior pituitary

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Abstract

Objective: Early growth response protein-1 (Egr-1)/nerve growth factor-induced A (also known as Zif-268, Krox-24, TIS8, ZENK) is a zinc-finger transcription factor which, although expressed in a range of organ systems, has been shown to be essential only in the maintenance of fertility through actions within the endocrine system. In the present study we have investigated the anatomical basis for actions of Egr-1 in the adult anterior pituitary gland.

Design: Using female rats and mice as experimental models, we have used immunocytochemical and microscopic analysis to make observations of the cellular and sub-cellular localization of Egr-1 protein.

Methods: Immuno-(Western) blotting was first used to characterize the anterior pituitary proteins detected by a commercially available Egr-1 antibody. Subsequently, the antibody was used both singly and in combination with an LH β -subunit antibody, for immunocytochemical localization studies in pituitary sections.

Results: The Egr-1 antiserum detected a single, major (primarily nuclear) 75 kDa protein band in Western blots of anterior pituitary extracts. Nuclear localization was confirmed by immunocytochemistry, which also demonstrated that the 75 kDa protein is localized to a minority sub-population of anterior pituitary cells in both rat and mouse. Dual immunocytochemical localization showed that the Egr-1 protein is primarily restricted to the nuclei of LH β -subunit-expressing cells.

Conclusions: Sub-cellular localization of Egr-1 to the nucleus is consistent with a direct role in transcriptional regulation of anterior pituitary function in the adult rat. Furthermore, a selective role in the physiological control of gonadotrophin gene expression is indicated.

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Introduction

Recent studies of the immediate early gene *egr-1*/NGFI-A (early growth response protein-1/nerve growth factor-induced gene A), which encodes the zinc-finger transcriptional regulator Egr-1, have focused attention on endocrine phenotypes. In two gene-targeting studies in mice (1, 2), mutant alleles have been shown to be associated with infertility that appears to be caused, perhaps primarily (see (2)), by a disorder of development in which gonadotrophs fail to express the luteinizing hormone β -subunit (LH β) pituitary hormone gene. These findings are consistent with *in vitro* studies of the LH β gene promoter which contains a functional Egr-1 DNA-binding site (3–5). We have recently extended this functional association by

describing correlations between Egr-1 activity, and the cyclical changes of the gonadotrophin axis in adult female rats (6).

A surprising aspect of the mouse 'knock-out' studies described above (see (2)) concerned the cellular compartmentalization of Egr-1 in wild-type mice. Thus, it was observed that Egr-1 was confined to the cytoplasm of anterior pituitary cells, whereas in many previous studies Egr-1 had been shown to be a nuclear protein, for example in brain (see (7)), which is consistent with both the presence of a nuclear localization domain (8) and the transcriptional regulatory activity of this zinc-finger protein (9–12). While there are precedents for transcriptional regulators residing in the cytoplasm (13), and cytoplasmic forms of Egr-1 have been observed in PC-12 cells (14), it

would be unusual for a particular factor to be wholly cytoplasmic in one cell type (pituitary), and nuclear in another (brain). In order to further our studies of Egr-1 function in the anterior pituitary, we have therefore conducted an investigation of Egr-1 localization in this tissue. We have used tissues from two species, in order both to substantiate previous findings in the mouse (2), and to contrast with the rat, which is the species of choice for our neuroendocrine studies. In addition, we have also investigated the extent of Egr-1 and LH β co-expression.

Materials and methods

Animals

Animal studies were conducted in accordance with both UK Home Office regulations and local ethical review. Adult (3–4 month), female Sprague–Dawley (CD) rats and CBA/J mice were maintained in standard laboratory conditions on a 14 h:10 h light:darkness cycle (lights on 05.00 h). Oestrous cycle stage was determined by microscopic analysis of vaginal washings. Animals were killed by cervical dislocation at 12.00 h on the day of proestrus, and pituitary glands were either frozen on dry ice (in embedding medium (Merck, Poole, Dorset, UK) for subsequent sectioning and immunohistochemistry), or prepared for protein extraction (anterior lobes only) as described (whole cell (15), nuclear (16)). Tissue and protein samples were stored at -70°C .

Immunoblotting

An Egr-1-specific rabbit polyclonal antiserum (Egr-1 (C19); Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used to probe Western blots of nuclear and whole cell protein fractions as described previously (15). Protein concentrations were determined by the method of Bradford (17). Molecular weight markers used were Broad Range Protein Marker (NEB, Beverly, MA, USA). The primary antiserum was used at a final dilution of 1:4000 (determined in preliminary studies), and secondary antiserum (horseradish peroxidase-linked donkey anti-rabbit; Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK) at 1:5000. Proteins were detected using chemiluminescence (HRPL kit; National Diagnostics, Atlanta, GA, USA).

Immunocytochemistry

Immunocytochemistry was performed on 8 μm sections of anterior pituitary, post-fixed in 4% paraformaldehyde in PBS (5 min), and permeabilized in methanol (-20°C , 2 min). Proteins were detected using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol, using diaminobenzidine (DAB) as chromogen.

Tissue sections were incubated with either diluted normal rabbit serum, Egr-1 antiserum, 1:400, (C19) or LH β antiserum, 1:1000 (NIDDK anti-rb β LH-IC-2; AFP22238790GPOLHB; National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda, MD, USA). Secondary antisera used were, for Egr-1, biotinylated anti-rabbit IgG (ABC kit) and for LH β , biotinylated anti-guinea pig IgG (BA7000; Vector). Following development of the chromogen, slides were counter-stained with Meyer's haematoxylin. The specificity of the Egr-1-associated staining was verified by immunoneutralization (overnight incubation of primary antisera in 10-fold (w/w) excess of peptide epitope; Santa Cruz). For dual localization of Egr-1 (nuclear) and LH β (cytoplasmic), the protocol was modified to incorporate simultaneous incubations with firstly the two primary antisera, and then the two secondary antisera. Control experiments verified that the use of two secondary antisera alone was not associated with cellular DAB staining. Quantitative cell counts were obtained from multiple microscopic fields (see Results) sampled from each of two different pituitary sections taken from three individual rats.

Results

In order to characterize the antiserum used in subsequent immunohistochemical analyses a Western blot of whole cell and nuclear anterior pituitary extracts from rat was performed (Fig. 1). Using the C19 antiserum, a single major immunoreactive band of approximately 70–75 kDa was detected, which was largely restricted to the nuclear fraction. A second, minor band of approximately 90 kDa was also observed, which was more similarly represented in whole cell and nuclear extracts. Longer autoradiograph

Figure 1 The C19 antiserum detects a primarily nuclear, approximately 75 kDa protein in rat anterior pituitary. Western blot of whole cell (wc) or nuclear (nuc) extracts (12 μg /lane) derived from rat anterior pituitary. The large arrow indicates the major 75 kDa protein band, and the small arrow indicates the position of the minor 90 kDa protein band. The position of molecular mass markers (NEB) is shown on the left.

Figure 2 *Egr-1* is primarily expressed in the nuclei of a sub-population of anterior pituitary cells of rat and mouse. Immunohistochemical analysis of anterior pituitary sections using DAB (brown) as chromogen to detect C19 (*Egr-1*)- and LH β -immunoreactivity, and Meyer's haematoxylin (blue/purple) as counter-stain. (A) Representative view of rat anterior pituitary showing localization of *Egr-1*-immunoreactivity in nuclei, magnification $\times 1000$. Arrow indicates a group of three *Egr-1*-positive cells surrounding an *Egr-1*-negative cell. (B) Representative view of mouse anterior pituitary showing localization of *Egr-1*-immunoreactivity in nuclei, magnification $\times 1000$. Arrow indicates a single *Egr-1*-positive cell. (C) Representative view of rat anterior pituitary showing cytoplasmic localization of LH β -immunoreactivity (arrow), magnification $\times 1000$. Note the absence of nuclear DAB staining. (D) Representative view of anterior lobe of rat pituitary showing co-localization of two *Egr-1*-positive nuclei with cytoplasmic LH β (arrow). A single *Egr-1*-positive nucleus (arrowhead) which is non-co-localized with LH β is also shown. Magnification $\times 1000$.

exposures did not reveal additional protein bands in the 55 kDa range (see below, data not shown). These findings contrast with our previous observations using another antiserum (588; Santa Cruz) in which two major *Egr-1*-immunoreactive bands of approximately 55 and 70 kDa were found (together with the minor 90 kDa band (6)). It has been suggested that the approximately 55 kDa protein represents a truncated form of *Egr-1*, which is consistent with the majority of studies that show full-length *Egr-1* to migrate at between 70 and 80 kDa on SDS-PAGE gels (e.g. 8, 14). Therefore, the C19 antiserum would appear to discriminate between these two forms, as demonstrated previously for other *Egr-1*-specific antisera. Consequently we selected the C19 antiserum for subsequent immunohistochemical studies, and, based on the size estimate obtained in the current study, have designated the protein as 75 kDa.

Consistent with the immunoblotting analysis described above, *Egr-1*-immunoreactivity was clearly observed as being nuclear in rat anterior pituitary tissue sections (Fig. 2A). Cells exhibiting nuclear *Egr-1* expression were confined to the anterior lobe; no DAB

staining was found in the intermediate lobe (apart from a layer at the anterior pituitary border), whereas only a diffuse light brown reaction product was observed in the neural lobe (data not shown). Following peptide neutralization of the C19 antiserum, the nuclear DAB staining in the anterior lobe cells was lost; in contrast, both the diffuse staining in the neural lobe, and the staining at the anterior/intermediate border, were not diminished, indicating an artefactual rather than genuine *Egr-1* expression in these areas (data not shown).

Nuclear *Egr-1* was localized to a sub-population of anterior pituitary cells, representing $11.4 \pm 0.9\%$ of the total (mean $\pm$ S.E.M., $n = 6$ ($\times 400$) fields selected from each of two sections taken from three individual rats). In horizontal sections of the anterior pituitary, the cells were distributed throughout the lobe although some areas contained a relative abundance of *Egr-1*-positive cells. Notably, the *Egr-1*-positive cells were often observed to be grouped (Fig. 2A, see below). In the mouse (Fig. 2B), very similar results were observed with *Egr-1*-positive nuclei being confined to a sub-population of anterior lobe cells. As in the rat,

immunoneutralization showed that nuclear Egr-1-immunoreactivity in the mouse anterior pituitary was also Egr-1 peptide epitope-specific (data not shown).

In order to attempt co-localization of Egr-1 with LH β in the rat, we first confirmed that our LH β antibody (see Materials and Methods) detected a sub-population of anterior pituitary cells with cytoplasmic localization (Fig. 2C). The diffuse cytoplasmic DAB reaction product obtained with the LH β antibody contrasted markedly with the dense nuclear DAB staining obtained with the Egr-1 antibody, and therefore co-localization using the same enzyme system and chromogen in a dual immunohistochemical analysis was feasible. Using this approach in the rat, we proceeded to show that Egr-1 was co-expressed with LH β , often in groups of cells (Fig. 2D). It was apparent that the majority of LH β cells exhibited nuclear Egr-1-immunoreactivity. This analysis also revealed that only a minority of Egr-1-positive cells (Fig. 2D) were LH β -negative ($13.4 \pm 1.6\%$ of total Egr-1 cells, mean $\pm$ S.E.M., $n = 18$ ($\times 400$) fields selected from two sections taken from three individual rats).

Discussion

The present study has been important in determining that the 75 kDa product of the *egr-1* gene is predominantly expressed in LH β -expressing cells of the adult female rat anterior pituitary. This finding is indicative of a selective functional association between the zinc-finger transcription factor Egr-1 and LH gene expression. Furthermore, both biochemical and immunocytochemical sub-cellular localization have clearly demonstrated that the Egr-1 protein is primarily found in the nucleus of both rat and mouse anterior pituitary cells, contrasting with the findings of a previous study, in mice, in which a wholly cytoplasmic localization for Egr-1 protein was evident (2). Although a cytoplasmic activity of Egr-1 could potentially be consistent with a role in transcriptional regulation, perhaps through binding of co-factors (8), the weight of evidence supports a classical, direct DNA interaction within the promoter region of the LH β gene (3–5, 18). The discrepancy between the findings of the present study and those of Topilko *et al.* (2), in which Egr-1 was shown to be cytoplasmic, may involve multiple variables including mouse strain (unlikely), immunohistochemical protocol, and primary antisera. In contrast to the present study, the previous study (2) did not report precise characterization of the antisera with Western analysis and peptide neutralization; the discrepancy cannot be resolved until such data are available. Our findings are important because a considerable body of research is now in agreement that Egr-1/NGFI-A is a necessary factor in the establishment of mammalian fertility (1, 2), through transcriptional up-regulation of the LH β gene (4). Our previous study (6) has indicated that this role is maintained in the adult, and possibly

extended to include a dynamic role in which cyclical changes in Egr-1 activity correlate with pre-ovulatory events. The adult expression pattern of Egr-1 demonstrated in the present study is consistent with a (nuclear) role in the regulation of LH β expression. Further studies involving an inducible, gonadotroph-specific knock-out of the *egr-1* gene will be required to establish this physiological role.

The demonstration that nuclear Egr-1 is largely confined to LH β cells in the adult rat suggests a primary functional association with the gonadotrophin axis. This result is both consistent with, and serves to explain, our previous study (6) in which cyclical changes in Egr-1 activity were observed in whole anterior pituitary extracts sampled during the rat oestrous cycle. Thus, it is now evident that these changes in Egr-1 were clearly observable in whole glands because of the absence of a non-cycling, background expression in a major alternative anterior pituitary cell population. The identity of the Egr-1-positive/LH β -negative cells has not been established in the present study, although it is clear that these cells are a minority population and could not represent a significant proportion of a major pituitary hormone cell group (19). The latter finding contrasts with expression in heterozygote *egr-1/lacZ* 'knock-in' mice in which β -galactosidase was observed in most growth hormone (GH), LH β , follicle-stimulating hormone- β and thyrotrophin- β cells (2); overall therefore, the reporter gene was expressed in a majority of anterior pituitary cells. Although co-localization studies in the mouse have not been performed in the present study, the relatively sparse distribution of (nuclear) Egr-1-positive cells in this species, which mirrors the distribution in the rat, clearly shows that nuclear p75 Egr-1 is not highly represented in a similar variety of pituitary cell types. A major, nuclear, role within the regulation of a second endocrine axis, in addition to the gonadotrophin, would therefore appear unlikely in the adult rat and mouse. This contrasts with a more generalized role for Egr-1 in the development of mouse anterior pituitary function, which appears to involve important effects on both the gonadotrophin and somatotrophin axes, and may reflect major changes in Egr-1 expression patterns during development (see (2)).

In conclusion, our demonstration of a selective co-localization of the nuclear p75 form of Egr-1 and the LH β in the adult rat has provided further evidence of an important role for this transcription factor in gonadotrophin expression, and therefore function.

At the same time, it should also be noted that recent studies have confirmed abundant expression of GH mRNA in a sub-population of gonadotrophs in adult rats (20). Although our present studies have shown Egr-1 to be primarily co-expressed with LH β -immunoreactivity, it is apparent, therefore, that this result does not imply a restriction to classical gonadotroph function.

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