Biochem. J. (1992) 287, 681–684 (Printed in Great Britain)

RESEARCH COMMUNICATION

Tissue distribution of mRNA for heparin-binding epidermal growth factor

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Heparin-binding epidermal growth factor (HB-EGF) is a recently identified member of the EGF family. Mature HB-EGF is processed from a larger transmembrane precursor which can itself act as a cell-surface receptor for the internalization of diphtheria toxin into eukaryotic cells. However, to date there is no information available on the distribution of HB-EGF in mammalian tissues. We have therefore used reverse-transcription PCR to analyse the expression of HB-EGF mRNA in a wide range of tissues. HB-EGF transcripts were detected in RNA isolated from 15 of the 22 tissues obtained from adult pigs, which is consistent with the ability of diphtheria toxin to affect many body tissues.

INTRODUCTION

The epidermal growth factor (EGF) family have a common ability to bind and activate the EGF-receptor tyrosine kinase. The family includes at least four mammalian polypeptides; EGF itself, transforming growth factor α (TGFα), amphiregulin and heparin-binding epidermal growth factor (HB-EGF) [1]. This last factor was discovered most recently and was purified from medium conditioned by a macrophage-like cell line [2]. HB-EGF is a polypeptide containing 86 amino-acid residues, the precursor of which is 208 amino acids in length and, like other members of the EGF family, includes a putative *N*-terminal signal peptide and a hydrophobic transmembrane domain [1,2]. Proteolytic processing of this precursor molecule releases mature HB-EGF which comprises a number of variants with *N*-terminal micro-heterogeneity [3]. The mature forms of HB-EGF can be *O*-glycosylated [3], and most closely resemble amphiregulin in overall structure [1,2,4].

In contrast with other members of the EGF family [1,5], there is currently no information available concerning the distribution of HB-EGF mRNA or protein in tissues and/or cell types other than macrophages. This is of particular significance, since the transmembrane HB-EGF precursor has been shown recently to be exploited by diphtheria toxin (DT) as a cell-surface 'receptor' responsible for the binding and internalization of the toxin into mammalian cells [6]. In this study, we have analysed the distribution of HB-EGF mRNA in a wide range of tissues obtained from adult pigs, as a first step towards understanding the role of this novel factor in normal cell regulation.

EXPERIMENTAL

Isolation of RNA

Large White pigs were either killed at a local slaughterhouse by stunning and exsanguination or at this Institute by intravenous injection of an overdose of barbiturate anaesthetic. Tissue samples were removed and processed immediately or snap-frozen in liquid nitrogen and stored at −70 °C until required. Total RNA was isolated by a single-step guanidine thiocyanate method [7].

Oligonucleotide primers

Oligonucleotide primers were synthesized on a Biosearch 8750 four-column DNA synthesizer (Biosearch, San Rafael, CA, U.S.A.). The HB-EGF primers were designed according to regions of the human cDNA coding sequence [2], taking into account codon degeneracy. For cloning purposes, the upstream and the two downstream primers contained 8-bp-long 5′-extensions incorporating either a *Bam*HI (5′-CGGGATCC-3′; upstream primer) or a *Xba*I (5′-GCTCTAGA-3′; downstream primers) restriction site. The upstream primer [5′-AAAAGA-AAGAA(G/A)AA(A/G)GG(C/A)-3′] represented nucleotides 538–555 of the human cDNA and was used both in the initial identification of the HB-EGF gene and in the tissue distribution analysis in conjunction with two different downstream primers. The first downstream primer [5′-TTC(A/T)CC-(G/A)TG(G/A)ATGCAGAA-3′] was used only in the initial identification of the HB-EGF gene and is the reverse complement of nucleotides 603–621. This primer pair predicts a 100 bp amplified fragment. The downstream primer that was used for the analysis of tissue distribution [5′-CCT(G/A)TG-(G/A)TACCT(G/A)AACAT-3′] is the reverse complement of nucleotides 808–825 and, in conjunction with the above upstream primer, predicts a 304 bp amplified fragment. This primer pair spans an intron which is conserved in genes of other members of the EGF family [5,8,9].

The β-actin upstream primer (5′-CTACAATGAGCTGCGT-GTGG-3′) was identical with nucleotides 192–211 of the mouse β-actin cDNA [10], while the downstream primer (5′-TAGC-TCTTCTCCAGGGAGGA-3′) was the reverse complement of nucleotides 622–641. This primer pair predicts a 450 bp fragment and spans a known intron, such that amplification of actin mRNA (as cDNA) may be distinguished from amplification of any contaminating genomic DNA.

Reverse-transcription PCR (RT-PCR)

First-strand cDNA was synthesized from total RNA (5 μg)

Abbreviations used: EGF, epidermal growth factor; HB-EGF, heparin-binding EGF; TGFα, transforming growth factor α; DT, diphtheria toxin; RT-PCR, reverse-transcription polymerase chain reaction; CNS, central nervous system.

The sequence reported in this paper has been submitted to the EMBL/Genbank Data Libraries under the accession number X67295.

using a First-Strand cDNA Synthesis kit (Pharmacia) and 50 pmol of the appropriate downstream primer. Reaction products were precipitated with 0.1 vol. of 4 M-NaCl and 2.5 vol. of ethanol and resuspended in 10 μl of water. Half of the cDNA was subjected to PCR amplification using a Techne PHC-1 programmable Dri-Block (Techne Ltd, Duxford, Cambs., U.K.). The amplification profile comprised 35 cycles: at 94 °C for 0.3 min (dissociation), between 50 °C and 65 °C (for different primer pairs) for 0.5 min (annealing) and 72 °C for 1.0 min (extension). The final cycle included a further 5 min at 72 °C for complete strand extension. Each reaction mixture contained 5 μl of cDNA, 50 pmol of each primer, 5 μl of 10 × PCR buffer (Promega), 2 μl of 5 mM-dNTPs, and water up to 50 μl. After overlaying with 50 μl of light mineral oil (Sigma), the reaction mixtures were heated to 94 °C for 5 min before the addition of 2.5 units of *Taq* polymerase (Promega).

Southern-blot analysis

Digested pig genomic DNA (10 μg) or aliquots (15 μl) of PCR reaction products were separated by electrophoresis on 0.9 % and 1.5 % (w/v) agarose gels respectively, and transferred to nylon membranes (Hybond-N from Amersham International, Amersham, Bucks., U.K.). Probes were radiolabelled by the random-primed DNA-synthesis method [11]. High-stringency hybridization and washing conditions were employed as previously described [12]. Hybridizing sequences were detected by autoradiography using Fuji RX X-ray film.

RESULTS

Identification of the HB-EGF gene in the pig genome

To determine whether the gene encoding HB-EGF was present in the pig genome, we used pig genomic DNA as a template for PCR, in conjunction with primers that were expected to lie within a single exon of the HB-EGF gene (see the Experimental section) [2,8]. On amplification, a band of the predicted size (100 bp) was evident on an ethidium bromide-stained agarose gel, and the nucleotide sequence of this product was found to be 85 % identical with the cDNA sequence for human HB-EGF [2] (see below and Fig. 3*a*).

To date, it is not known whether HB-EGF is encoded by a single gene or by a multigene family. To answer this question, the 100 bp amplified fragment was radiolabelled and used to probe restriction-endonuclease digests of pig genomic DNA. Under high-stringency hybridization and washing conditions, only one hybridizing band was detectable in each digest (Fig. 1), indicating that HB-EGF is present as a single-copy gene in the pig genome, as previously reported for the related factor EGF [13].

Tissue expression of HB-EGF mRNA

To investigate the tissue distribution of HB-EGF mRNA, we used the highly sensitive technique of RT-PCR in conjunction with oligonucleotide primers (see the Experimental section) corresponding to sequences that traverse an exon/intron boundary that is conserved in EGF [8], TGFα [9] and amphiregulin [5]. An amplified product of the predicted size (304 bp) was visible on an ethidium bromide-stained agarose gel when total RNA isolated from skin, midbrain, cerebellum, hypothalamus, cerebral cortex, bulbourethral gland, lung, heart (ventricle), kidney, prostate, seminal vesicle and testis was used as the template for the RT-PCR (Fig. 2*a*), indicating the presence of HB-EGF transcripts in these tissues. While total RNA isolated from lymph node, thymus and spleen failed to yield an amplified product that was clearly visible on an ethidium bromide-stained agarose gel (Fig. 2*a*), a band of the expected size was detected

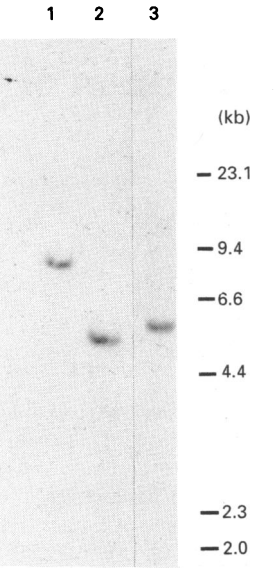

Fig. 1. Southern blot of pig genomic DNA

Pig genomic DNA (10 μg/lane) was digested with *Hin*dIII (lane 1), *Bam*HI (lane 2) and *Bgl*II (lane 3), electrophoresed through a 0.9 % (w/v) agarose gel and transferred to a nylon membrane. The blot was hybridized with a radiolabelled 100 bp pig HB-EGF DNA probe, washed under conditions of high stringency and exposed to X-ray film for 5 days with an intensifying screen at −70 °C. The positions of *Hin*dIII-digested λDNA markers (in kb) are indicated.

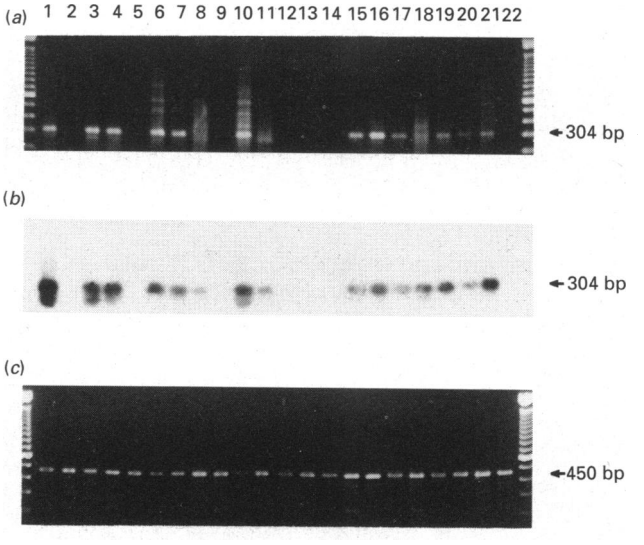

Fig. 2. Distribution of HB-EGF mRNA in pig tissues

Total RNA was isolated from skin (lane 1), pituitary (2), midbrain (3), cerebellum (4), olfactory bulb (5), hypothalamus (6), cerebral cortex (7), lymph node (8), thyroid (9), bulbourethral gland (10), thymus (11), duodenum (12), pancreas (13), liver (14), lung (15), heart (ventricle) (16), kidney (17), spleen (18), prostate (19), seminal vesicle (20), testis (21) and submaxillary gland (22) and submitted to RT-PCR using HB-EGF-specific [(*a*) and (*b*)] and β-actin-specific (*c*) primers, and electrophoresed through a 1.5 % agarose gel (*a*, *c*) Ethidium bromide-stained agarose gel. (*b*) The gel from (*a*) was transferred to a nylon membrane and hybridized with a radiolabelled 100-bp-long pig genomic HB-EGF DNA probe. After washing at high stringency, the blot was exposed to X-ray film for 4 h with an intensifying screen at −70 °C. Molecular-mass markers are a 100 bp ladder (Gibco, BRL, Paisley, Scotland, U.K.). The sizes (bp) of the predicted amplified products are indicated.

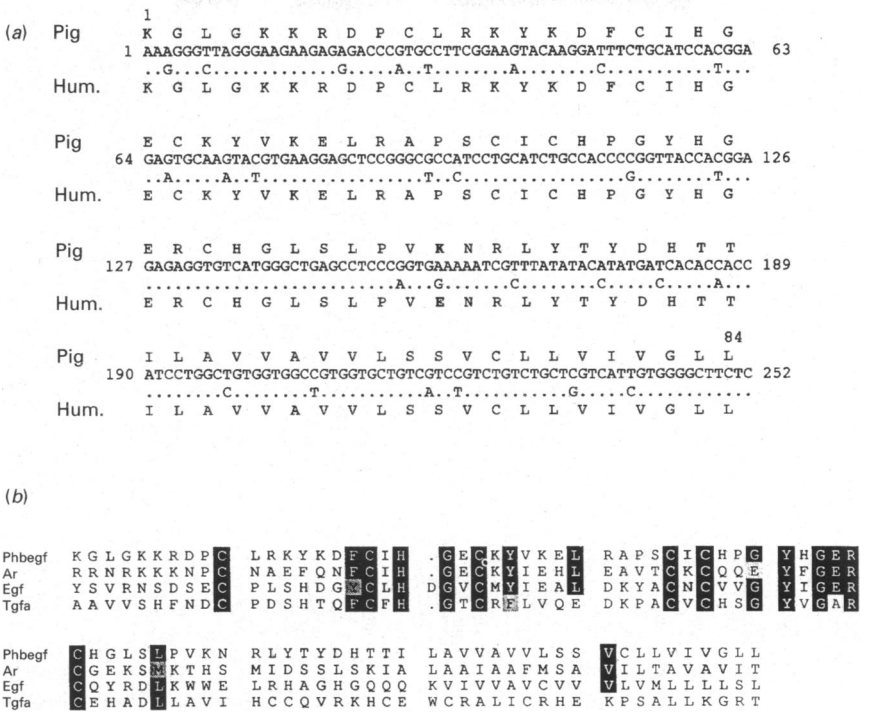

Fig. 3. Sequence comparisons of pig HB-EGF with human HB-EGF (*a*) and with other members of the EGF family of polypeptides (*b*)

(*a*) Nucleotide sequence of the cloned 252 bp (discounting primer sequences) of the pig HB-EGF cDNA. Changes from the human HB-EGF cDNA [2] are indicated. The deduced pig HB-EGF amino-acid sequence is shown above the nucleotide sequence and the human HB-EGF amino-acid sequence below. The single amino-acid alteration is indicated in bold type. (*b*) Comparison of the deduced partial amino-acid sequence of pig HB-EGF (Phbegf) with the relevant regions of amphiregulin (Ar) [5], EGF (Egf) [8] and TGFα (Tgfa) [23], with alignments centred upon the six conserved cysteine residues. Only residues appearing in three or more proteins are indicated by solid boxes. Stippled boxes indicate conservative changes. Points indicate a gap introduced to maximize sequence similarity. The standard one letter code for amino acids has been used.

after transfer to a nylon membrane and probing with a radiolabelled 100 bp HB-EGF genomic DNA probe (Fig. 2*b*). This suggests that there are lower, but detectable, levels of HB-EGF mRNA present in these three tissues. No HB-EGF-specific amplified products were detected when total RNA isolated from pituitary, olfactory bulb, thyroid, duodenum, pancreas, liver or submaxillary gland was used as the template for the RT-PCR (Figs. 2*a* and 2*b*). As a control for the integrity of the RNA, the same RNA samples were also analysed for β-actin expression. Using RT-PCR and primers specific for β-actin, all samples gave an amplified product of the expected size (450 bp, Fig. 2*c*). All RNA samples that were positive for HB-EGF by RT-PCR were further analysed by Northern blotting. However, we were unable to detect HB-EGF transcripts in total RNA (20 μg) derived from any of the tissues studied, suggesting that levels of HB-EGF mRNA may be relatively low, as has been observed for TGFα [1,14].

No amplification products were detected when either the cDNA, one of the primers, or the reverse transcriptase was omitted from the reaction, or when cDNA was replaced with 50 ng of genomic DNA. The latter observation demonstrates that an exon/intron boundary is present within this region (between nucleotides 621 and 808) of the HB-EGF gene, as found in other members of the EGF family [5,8,9]. To verify that the 304 bp amplified product encoded HB-EGF, amplified DNA fragments derived from several tissues were subcloned into pBluescribe-M13$^+$ and their nucleotide sequence determined (Fig. 3*a*). Discounting the primer sequences (52 bp), there is 89% sequence similarity between the cloned 252 bp sequence and human HB-EGF cDNA [2]. Furthermore, the 84 amino-acid residues encoded by this region (which includes over 70% of the mature peptide) contain only a single alteration (Glu-53 changed to Lys) when compared with the human HB-EGF sequence (Fig. 3*a*). In contrast, similarity to other members of the EGF polypeptide family is more limited, with conservation only of those residues known to be important for high-affinity binding to the EGF receptor (Fig. 3*b*). These results demonstrate that the cloned sequence encodes pig HB-EGF and that the amino-acid sequence of this factor is highly conserved between species.

DISCUSSION

HB-EGF was originally identified in human macrophages and in a human macrophage-like cell line [2]. However, there is no published information concerning other sites of synthesis of this factor *in vivo*. A detailed knowledge of the sites of expression of a novel growth factor is an important pre-requisite in beginning to define its physiological function(s). From the results presented here, the principal sites of HB-EGF synthesis appear to include skin, kidney, lung, heart (ventricle), male reproductive organs and the central nervous system (CNS). Interestingly, the related factors, EGF and TGFα, have also been identified in skin [1,15], kidney [16,17], lung [1,17] and male reproductive tissues [13,18], but not in heart [19] (T. J. Vaughan, unpublished work). We were also able to detect HB-EGF expression in various regions of the adult CNS. HB-EGF may serve similar functions in the brain to those proposed for EGF and TGFα in the regulation of CNS growth, maintenance and differentiation [20].

Since HB-EGF is synthesized by macrophages [2], it is possible that macrophages resident within a tissue may result in HB-EGF mRNA being detected by the RT-PCR. Indeed, a number of the tissues analysed (skin, liver, brain, lung, spleen, thymus and lymph node) contain macrophages in the absence of tissue damage [21]. However, tissues such as the spleen and lymph

node, which are comparatively macrophage-rich, give a signal that is detectable only after hybridization with a ^{32}P-labelled probe (Fig. 2*b*). Hence, those signals that are clearly visible on an ethidium bromide-stained agarose gel (Fig. 2*a*) are unlikely to be macrophage-derived. However, to confirm this, future studies will be directed at localizing the sites of HB-EGF biosynthesis in expressing tissues using *in situ* hybridization and immunocytochemistry.

DT inhibits protein synthesis, is lethal for many animal species, and is cytotoxic for a number of cultured cell lines. While the causative agent of diphtheria, *Corynebacterium diphtheriae*, is localized to the upper respiratory tract, the exotoxin released by the pathogen can disrupt the normal physiology of many body organs, including the heart, kidney, lung and nervous system, although the tissues affected may vary slightly from one species to another [22]. The cellular uptake of DT is mediated through a specific cell-surface receptor, recently identified as the HB-EGF transmembrane precursor [6]. The results presented here indicate that HB-EGF mRNA is present in a wide range of body tissues, and is consistent with the observed DT-sensitivity of many tissues and cell types.

We are grateful to Tony Corps for helpful comments on the manuscript, and to Richard Binns and Derek Booth for their help in obtaining tissue samples. This work was supported by a grant from the A.F.R.C. Pig Science Programme.

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Received 29 July 1992/28 August 1992; accepted 2 September 1992