Journal of Chromatography, 491 (1989) 49–60
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4729

RAPID CHROMATOGRAPHIC ISOLATION AND IMMUNOBLOT CHARACTERIZATION OF IMMUNOREACTIVE FIBROBLAST GROWTH FACTOR-RELATED POLYPEPTIDES FROM VARIOUS TISSUES

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(First received December 19th, 1988; revised manuscript received February 14th, 1989)

SUMMARY

Procedures to rapidly isolate fibroblast growth factor (FGF)-like activity from a number of tissue sources (lung, plasma, brain, ovary, corpus luteum, pituitary, chondrosarcoma) of bovine, porcine or rat origin are described. In addition, immunoblotting experiments using well characterized and specific rabbit polyclonal anti-fibroblast growth factor β (anti-FGF-β) sera have been performed. Besides documenting the first report of the isolation of FGF-β from bovine lung and plasma, these studies provide evidence for the existence of higher-molecular-mass proteins with FGF-β-like immunoreactivity. For example, in addition to new truncated forms of the acidic and basic FGF (FGF-α and FGF-β), respectively, other higher-molecular-mass immunoreactive proteins were detected in bovine, pig and rat brain, and in rat chondrosarcoma. The tissue distribution of these immunoreactive proteins and their competitive inhibition characteristics mitigate against the possibility that the polyclonal antisera are cross-reacting non-specifically with common cellular proteins. Rather, the data suggest that the immunoblotting technique is either detecting other proteins structurally related to FGF-β or alternatively FGF-β is strongly bound to specific carrier proteins (e.g. heparan sulphate proteoglycan fragments) associated with their transport and recognition at the cellular level.

INTRODUCTION

Since the initial purification of the acidic and basic forms of fibroblast growth factor (FGF-α and FGF-β), respectively, from bovine brain- and pituitary-related [1–4] proteins have been identified in other tissues [5,6]. Considerable heterogeneity at the amino terminus of this polypeptide mitogen has been ob-

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served from primary sequence studies. Variants of the mature, 146-amino-acid residue FGF-β, [1–146]FGF-β, include a 15-amino-acid truncated polypeptide, des[1–15]FGF-β, from ovarian, kidney and adrenal sources [6–8], a 4-amino-acid truncated form, des[1–4]FGF-β, from bovine pituitary sources [9] and an 8-amino-acid extended form from the bovine pituitary and human prostate [10,11]. Similarly FGF-α has also been reported to exist as a 6-amino-acid truncated form, des[1–6]FGF-α [12], whilst an extended form (15 amino acids) has been found in the prostate [13].

A number of previous studies have established that some amino terminal truncated forms of FGF-α or FGF-β, i.e. the des[1–15]FGF-β, arise as a general consequence of non-specific proteolytic degradation [10,11]. The presence of protease inhibitors such as leupeptin, pepstatin and phenylmethylsulfonyl fluoride suppress this cleavage leading to the isolation, for example, from pituitary of an amino terminal extended form of the FGF-β polypeptide as predicted from the cDNA sequence [14]. However, in other studies with different tissues, truncated forms of FGF-α or FGF-β have been purified in the presence of protease inhibitors [9,15]. These observations suggest that tissue-specific mechanisms also exist by which precursor forms are processed by specific proteases to give the mature, truncated but biologically active polypeptides. Examples of this phenomenon are already well documented in the literature associated with the post-translational processing of the neuroendocrine polypeptide hormones and include the processing of a somatostatin precursor to either somatostatin-14 or -28 in various tissues [16,17], the production of cholecystokinin-33 in the intestine but cholecystokinin-12, -8 and -4 in the brain [18], the processing of proglucagon to either glicentin (M_r 11 000) or glucagon (M_r 3500) in the intestine or pancreas, respectively [19], and the differential cleavage of proopiomelanocorticotropin (POMC) in the anterior and intermediate lobe of the pituitary and the brain [18]. As part of studies on the structure and function of the basic and acidic forms of FGF, we have used a multi-stage protocol for the isolation of FGF and other proteins interacting with immobilized heparin. The possible relationship of isolated proteins to FGF-β was established by immunoblotting with specific polyclonal antibodies raised to des[1–4]FGF-β. This study reports the first isolation of FGF-β from bovine lung and plasma and the identification of a number of tissue-specific proteins, other than the mature form of FGF-α or FGF-β, which adsorb to immobilised heparin and which cross-react with specific antibodies to [1–146]FGF-β.

EXPERIMENTAL

Purifications

The M_r 17 500 form of FGF-β and other heparin-binding proteins were purified from bovine pituitary, bovine brain, rat brain, pig brain, rat chondrosarcoma, bovine lung, bovine ovary, bovine corpus luteum and bovine plasma as

previously described [9]. All procedures were performed at temperatures below 4°C. A 1-kg amount of tissue was homogenized in 2 l of 0.15 *M* $(NH_4)_2SO_4$ containing 1 m*M* EDTA, 2 m*M* phenylmethylsulfonyl fluoride, 5.0 mg leupeptin and 5.0 mg of pepstatin. Proteins were extracted at pH 3.5 and recovered by precipitation with 4.1 *M* $(NH_4)_2SO_4$ after an initial fractionation cut at 2.2 *M* $(NH_4)_2SO_4$. After desalting, the protein fraction was loaded from a pooled reservoir onto a cation-exchange chromatographic column of carboxymethyl (CM)-Sephadex C-50 at a flow-rate of 1.0 ml/min. Typically, a 500-ml bed volume was used (22 cm×5 cm I.D. Pharmacia K50/30 column) equilibrated with 0.1 *M* NaH_2PO_4, 0.1 *M* NaCl, pH 6.0. Bound proteins were eluted with 0.1 *M* NaH_2PO_4, 1.0 *M* NaCl, pH 6.0. Modified procedures were used for the rat chondrosarcoma and bovine plasma extracts. Rat chondrosarcoma homogenate or bovine plasma was acidified to pH 3.5, clarified by centrifugation, the pH readjusted to pH 6.0, recentrifuged and the supernatant loaded directly onto the CM-Sephadex C-50 ion-exchange column.

Pooled fractions of the CM-Sephadex C-50 1.0 *M* NaCl protein peak were diluted 2:3 (v/v) with 10 m*M* Tris–HCl pH 7.0, the pH adjusted to pH 7.0 with 1 *M* NaOH and the bulk feed stock loaded onto a heparin-Sepharose column (Pharmacia) typically of 20 ml bed volume (10 cm×1.6 cm I.D. Pharmacia K16/20 column), equilibrated with 10 m*M* Tris–HCl, 0.6 *M* NaCl, pH 7.0, at a flow-rate of 0.5 ml/min. The column was eluted with 75 ml of 10 m*M* Tris–HCl, 0.8 *M* NaCl, pH 7.0, then step-wise with Tris–HCl, pH 7.0 buffer containing 1.1 *M* NaCl and then 2.0 *M* NaCl or by a 1.1–2.0 *M* NaCl gradient. The flow-rate for desorption used was 0.5 ml/min.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The purification of the M_r 17 500 form of FGF-β and other heparin-binding-related polypeptides was monitored by analytical SDS-PAGE using vertical 12.5% polyacrylamide gels according to the method of Laemmli [20] in the presence of 2-mercaptoethanol. Protein bands were visualized by a silver-staining method [21].

Immunoblotting of protein preparation with FGF antiserum RO185

Aliquots (4 ml) of heparin-Sepharose chromatography eluents (1.1–2.0 *M* NaCl) were concentrated to 100 μl with Centricon-10 microconcentrators (Amicon) and 30-μl aliquots subjected to SDS-PAGE under reducing conditions. Proteins were then electroeluted from the gel onto a Zeta-Probe membrane (Bio-Rad) over 5 h at 200 mA in a Trans-Blot cell (Bio-Rad) filled with 15.6 m*M* Tris, 120 m*M* glycine, pH 8.3. Non-specific binding sites on the membrane were blocked with 10% bovine serum albumin (BSA) in 20 m*M* Tris, 500 m*M* NaCl, pH 7.5 at 45°C overnight. The transferred proteins were reacted with 100 ml of a 1:1000 dilution of a rabbit polyclonal antiserum RO185 in 2% BSA, 20 m*M* Tris, 500 m*M* NaCl, 0.05% Tween 20, pH 7.5 for 2 h at

room temperature. The specificity and binding capacity of the RO185 antiserum has been described elsewhere [9]. The RO185 antiserum specifically and saturably binds ≥35% of bovine [^{125}I]FGF-β at a dilution of 1:3000. Subsequent experiments have shown that the antiserum cross-reacts with rat and porcine β-FGF. Following washing for 20 min with four changes of solution with 20 m*M* Tris, 500 m*M* NaCl, 0.05% Tween 20, pH 7.5, the membrane was transferred to a 100-ml solution of 1:3000 dilution of affinity-purified goat anti-rabbit Ig (H+L) (human IgG absorbed) conjugated to alkaline phosphatase (Bio-Rad). Detection of the bound antibody conjugate was achieved by reaction with a substance mixture consisting of 30 mg of nitroblue tetrazolium (NBT) (Sigma) dissolved in 1 ml of 70% N,N-dimethylformamide and 15 mg of 5-bromo-4-chloroindoxylphosphate (BCIP) (Sigma) dissolved in 1 ml of N,N-dimethylformamide in 100 ml of 0.1 *M* $NaHCO_3$, 1.0 m*M* $MgCl_2$, pH 9.8.

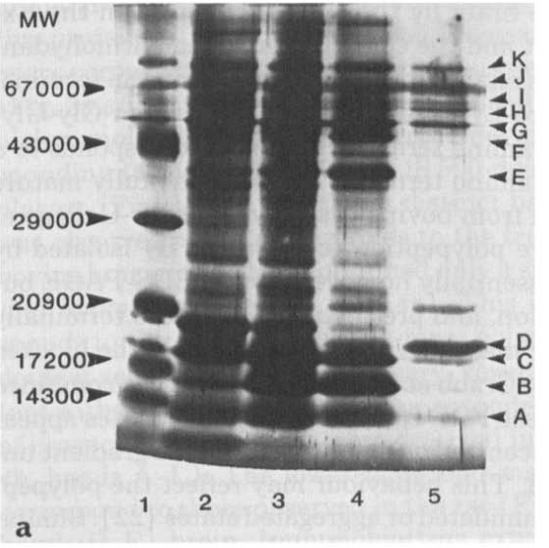
RESULTS

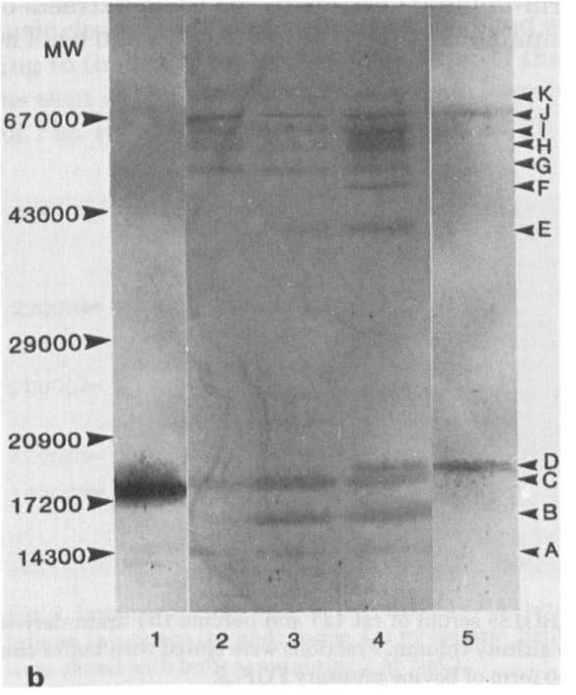
Proteins were extracted from a number of tissues from different mammalian species by the sequential application of cation-exchange chromatography and heparin affinity chromatography. To establish the relationship of the proteins to the mature M_r 17 500 form of basic FGF-β, immunoblotting was performed using a specific polyclonal raised to des[1–4]FGF-β [9]. Fig. 1 shows the SDS-PAGE of proteins eluted from a heparin-Sepharose column which has been loaded with the retained peak from the CM-Sephadex C-50 chromatographic fractionation of bovine brain-derived proteins. Unlike our previous studies describing the isolation of the M_r 17 500 form of FGF-β by this two-step procedure from bovine pituitary extracts [9], it is evident from the electropherogram that a large number of heparin-binding proteins encompassing the M_r range 14 300–67 000 can be recovered from bovine brain extracts (bands A–K, Fig. 1a). Lanes 2, 3, 4 and 5 correspond to proteins recovered from the 0.8, 1.1, 1.5 and 2.0 *M* NaCl batch elution steps, respectively, from the heparin affinity

Fig. 1. (a) SDS-PAGE of bovine brain-derived proteins eluted from a heparin-Sepharose affinity column by buffer containing 0.8–2.0 *M* NaCl. Elution was by gradient mode. Lanes 2, 3, 4 and 5 correspond to the 0.8–1.1, 1.1–1.5, 1.5–2.0 and 2.0 *M* NaCl fractions, respectively. A 4-ml aliquot of the eluted pools was concentrated to 100 μl, diluted in three volumes of sample buffer containing SDS and 2-mercaptoethanol, boiled at 100°C for 2 min, and 100 μl of the mixture were electrophoresed. Proteins were visualized by silver staining. Protein standards (chick lysozyme, myoglobin, swan lysozyme, carbonic anhydrase, ovalbumin and BSA) consisted of 150 ng of each protein. (b) Immunoblotting of bovine brain derived-proteins corresponding to those shown in (a). Proteins separated by electrophoresis were electrotransferred to a Zeta-Probe membrane over a 5-h period at 200 mA. Following incubation in blocking buffer containing 10% BSA, the membrane was incubated in a 1:1000 dilution of the polyclonal RO185 anti-FGF-β serum for 3 h. Specifically bound antibody was visualized with affinity-purified goat anti-rabbit IgG alkaline phosphatase conjugate using nitroblue tetrazolium–5-bromo-4-chloroindoxyl phosphate as the substrate system. Lane 1 is the M_r 17 500 form of bovine pituitary FGF-β.

chromatographic column. Immunoblotting of these proteins revealed many to be immunologically related to the M_r 17 500 form of FGF-β as indicated by their cross-reaction with the specific anti-serum RO185 (Fig. 1b).

Application of reversed-phase high-performance liquid chromatographic





(RP-HPLC) techniques permitted the further resolution of these brain-derived heparin-binding proteins. In particular, RP-HPLC protocols, previously developed [22] in this laboratory, enabled the isolation of homogeneous preparations of bovine FGF-α and FGF-β from different tissue sources. Twenty cycles of automated Edman degradation of the amino terminal region of the component A isolated from bovine brain by the procedures given in the Experimental section were carried out and the corresponding phenylthiohydantoin (PTH)-amino acid derivatives analysed. The determined amino terminal sequence was Asn-Tyr-Lys-Lys-Pro-Lys-Leu-Leu-Tyr-Cys-Ser-Asn-Gly-Gly-Tyr-Phe-Leu-Arg-Ile-Leu..... This amino terminal sequence corresponds to a 6-amino-acid truncation from the amino terminal region of the fully mature FGF-α form as previously isolated from bovine brain by Gimenez-Gallego et al. [12]. A further immunoreactive polypeptide (component B) isolated by RP-HPLC [23] was found to be essentially homogeneous by SDS-PAGE but failed to undergo Edman degradation, and presumably was amino terminally blocked. On the basis of molecular mass this protein is probably the blocked form of FGF-β, previously noted by Crabb et al. [13]. Based on the combined RP-HPLC and PAGE analyses, these FGF-α and FGF-β polypeptides appear to be eluted at several different concentrations of the acetonitrile gradient under the RP-HPLC conditions used. This behaviour may reflect the polypeptides in various conformational, deamidated or aggregated states [22]. Similar RP-HPLC behaviour has previously been described for other protein hormones. An examination of heparin-binding proteins in the brain extracts of the rat and pig revealed unique immunoblotting patterns (Fig. 2a and b). The

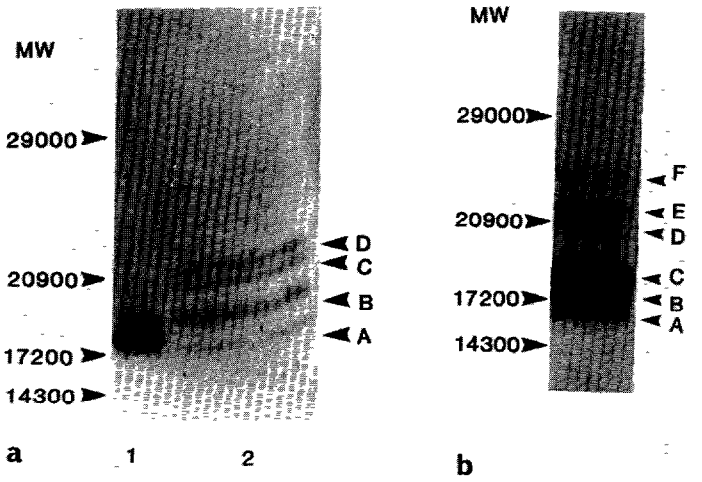


Fig. 2. Immunoblotting with polyclonal RO185 serum of rat (a) and porcine (b) brain-derived protein eluted from a heparin-Sepharose affinity column. Fractions were eluted with buffer containing 2 *M* NaCl. Lane 1 is the M_r 17 500 form of bovine pituitary FGF-β.

high-molecular-mass species observed with bovine brain extracts (Fig. 1a, E–K) were not observed with these species. However, both the rat and the pig brain preparations contained two immunoreactive heparin-binding proteins of apparent M_r 20 000 and 22 000, respectively (see Fig. 2a, bands C and D and Fig. 2b, bands D and E). Neither of these proteins were present in the bovine preparation. With the porcine brain preparation an additional immunoreactive protein of M_r 25 000 was also observed (Fig. 2b, band F). In the bovine ovary corpus luteum preparation immunoreactive proteins of 17 500 and 18 500 (Fig. 3a and b, bands B and C, respectively) were observed but not the other higher-molecular-mass immunoreactive species. These two proteins, corresponding to the M_r 17 500 and 18 500 doublet, were also observed in bovine plasma (Fig. 3c). In addition a distinct band (Fig. 3c, band B) of M_r 14 300 was observed which corresponds to the truncated form of acidic FGF-α. The bovine lung preparation contained only a single immunoreactive form of FGF-β of apparent M_r 18 500 (Fig. 4a). This immunoreactive polypeptide corresponds to the higher-molecular-mass polypeptide of the M_r 17 500 and 18 500 doublet seen in bovine brain, ovary, corpus luteum and plasma and is consistent with the 8-amino-acid residue extended form of [1–146]FGF-β. A number of cross-reactive proteins were detected in rat chondrosarcoma extracts (Fig. 4b, bands A–L). The lower-molecular-mass species (bands A–D) appear to correspond to those observed in the rat (Fig. 2b, bands A–D) and pig (Fig. 2b, bands B–E) brain. Immunoblotting experiments with chromatographically fractionated bovine pituitary preparations usually reveal the existence of only a single immunoreactive polypeptide band with a molecular mass corresponding to the lower-molecular-mass form of the FGF-β (i.e. the M_r 17 500 form) as seen in the various tissue sources described above (for example see lane 1 of Fig. 1b). However, in one purified batch of polypeptides related to FGF-β

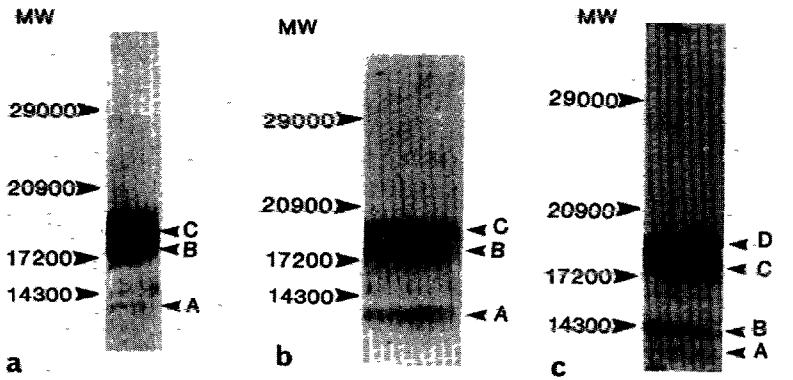


Fig. 3. Immunoblotting with the polyclonal RO185 serum of proteins isolated from bovine corpus luteum (a), ovary (b) and plasma (c) by heparin-Sepharose affinity chromatography. Fractions were eluted with buffer containing 2 *M* NaCl

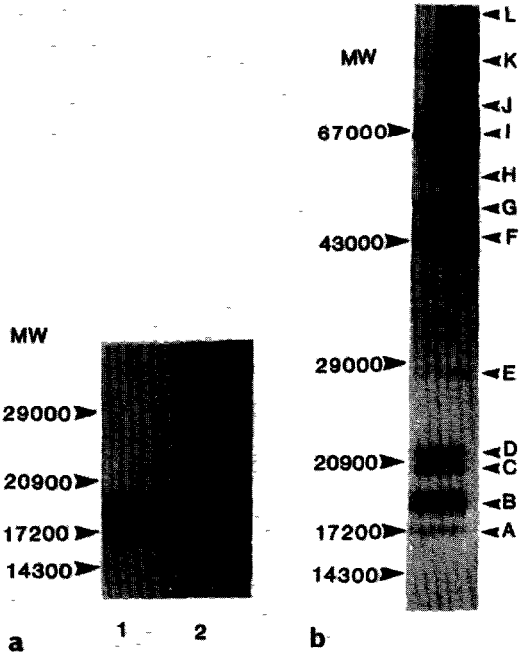


Fig. 4. Immunoblotting with polyclonal RO185 serum of proteins isolated from bovine lung (a) and rat chondrosarcoma (b) by heparin-Sepharose affinity chromatography. Lane 1 is the M_r 17 500 form of bovine pituitary FGF-β.

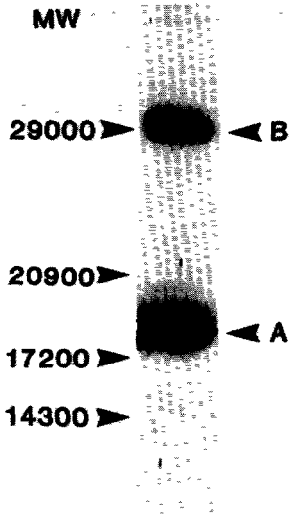


Fig. 5. Immunoblotting with polyclonal RO185 serum of proteins isolated during purification from one batch of bovine pituitary revealing the existence of second high-molecular-mass form not obtained with other purification batches 12 as can be seen in lanes 1 of Fig. 1b, 2a and 4a. A period of twenty months elapsed between these two purifications during which the pituitaries were stored at −20°C.

from a bovine pituitary preparation a second higher-molecular-mass immunoreactive protein of approximately M_r 29 000 which competitively cross-reacts with the specific FGF-β RO185 antiserum and is equipotent in various mitogen assays was also isolated (Fig. 5, band B). The difference between this and other pituitary preparations used in these studies is related to the length of time taken for the collection of fresh pituitaries and their storage at $-20°C$.

DISCUSSION

Based on the described protocols, heparin-binding proteins with FGF-like activity have been rapidly isolated from a number of tissue sources (lung, brain, ovary, corpus luteum, pituitary, chondrosarcoma and plasma) of bovine, porcine or rat origin. Immunoblotting of heparin-binding proteins isolated from various tissues has revealed a number of proteins, distinct from the mature, pituitary-derived 146-amino-acid residue FGF-β form originally used to raise in rabbits the polyclonal specific FGF-β antiserum. These proteins were found, in particular, in extracts of bovine, porcine and rat brain and rat chondrosarcoma. The bovine brain contained at least eleven immunoreactive polypeptide proteins ranging in apparent molecular mass from M_r 14 300 to 70 000. In the rat chondrosarcoma twelve immunoreactive species ranging in molecular mass from approximately M_r 17 200 to 90 000 were also observed. However, in bovine ovary, corpus luteum and plasma only two FGF-β species of M_r 17 500 and 18 500 were observed. In the rat and porcine brain in addition to these M_r 17 500 and 18 500 FGF-β species two other polypeptides of approximately M_r 22 000 and 25 000 were observed. In the bovine lung a single immunoreactive FGF-β of approximately M_r 18 500 was detected. Interestingly, one batch of FGF-related polypeptides purified from bovine pituitaries, which for ten other preparations has only led to the isolation of a single M_r 17 500 polypeptide, contained an immunoreactive protein of approximately M_r 29 000. Similar polydispersity in terms of molecular-mass distribution for FGF-β-related polypeptides has been observed by Mormede et al. [24] using high-performance size exclusion chromatography.

Although many proteins arise by post-translational processing from high-molecular-mass precursors [16–19], the existence of high-molecular-mass FGF-related proteins would appear to be incompatible with the reported cDNA sequences for FGF-α or FGF-β. Recent cDNA studies have indicated that the precursor forms have only a 15- or 9-amino-acid leader sequence, respectively [14]. Several explanations that account for the presence of these immunoreactive proteins can be proposed. Firstly, the observed chromatographic and electrophoretic behaviour of the high-molecular-mass forms of these proteins could represent non-reducible dimeric or oligomeric forms. Such behaviour has been previously noted for other protein hormones, e.g. growth hormone and inhibin [25]. However, it is unlikely that the immunoreactive proteins of ap-

proximately M_r 22 000 and 25 000 detected specifically in the rat and porcine brain or the rat chondrosarcoma preparations represent aggregates (of degraded forms) of FGF-β. With regard to the high-molecular-mass species (e.g. above M_r 25 000) the results do not exclude the possibility that non-reducible molecular species, derived from pituitary preparations, are aggregates involving different cleavage products which have arisen either biologically or by artifact due to the existence of tissue-specific proteases. The higher-molecular-mass forms seen in the bovine brain and chondrosarcoma could similarly represent aggregated, oligomeric forms. Alternatively, the immunoreactivity observed with the higher-molecular-mass proteins could arise from the presence of common epitopes with sequence or three-dimensional regions homologous to FGF-β which therefore cross-react with the polyclonal antibodies used in these and other immunoblotting experiments. This situation would be analogous to other observations on the homology found between regions of the low-density-lipoprotein receptor, epidermal growth factor (EGF) precursor and proteins of the blood clotting system, namely factors IX, X and protein C [26]. Other examples of physiologically unrelated proteins where limited sequence homology results in fortuitous immunoreactivity include a high-molecular-mass placental protein with β-endorphin- and adenocorticotropin-like immunoreactivity. Subsequent studies by Julliard et al. [27] have shown that this immunoreactive protein is a fragment of the heavy chain of the human immunoglobulin IgG1, with internal sequence regions (e.g. the sequence ...Ser^{364}-Leu-Thr-Cys-Leu-Val-Lys-Gly-Phe-Tyr-Pro-Ser-Asp-Ile^{377}...) exhibiting up to 40% homology with the antigenic determinant of β-endorphin (e.g. ...Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile...). Similarly, a unique protein which cross-reacts with antiserum against hFSH has been isolated from porcine follicular fluid. This protein was also recognized by a monoclonal antibody to human follicotropin (hFSH), indicating that distinct proteins with at least one common epitope can occur [28]. The possibility that these high-molecular-mass forms are artefacts of the isolation or detection procedures associated with the use of specific antisera can be discounted on the basis of quantitative results obtained with hyperimmune and control non-immune serum reported in our previous studies and the fact that the individual protein species are confined to specific tissue types and particular species. If, for example, the polyclonal antibodies present in the RO185 serum were interacting in a low-affinity fashion to particular ubiquitous cellular proteins then similar Western blot transfer patterns would be expected for all tissues. Furthermore, the possibility that the partially characterised proteins as detected by the immunoblotting technique are structurally related to FGF-β, besides their evident immunological cross-reactivity, is also suggested by similarities in their physicochemical properties as revealed by their adsorption characteristics to cation-exchange and heparin affinity chromatographic supports. Recent reports that FGF-β exhibits considerable homology to the putative pro-

teins coded by the hst and int-2 oncogenes [29] also raises the possibility that the detected proteins correspond to derivative products of these proto-oncogene precursors. The hst and int-2 oncogenes code for proteins consisting of 206 and 245 amino acids, respectively [30,31]. Polypeptide forms of these coded putative proteins would correspond in molecular mass to the M_r 20 000–27 000 polypeptides observed in the rat and pig brain and the rat chondrosarcoma preparations. It is obvious that partial amino acid sequencing of these protein species is required to answer these intriguing questions.

ACKNOWLEDGEMENTS

These studies have been supported by grants to M.T.W.H. from the National Health and Medical Research Council of Australia and the Australian Tobacco Research Foundation. M.G. is a Monash Postgraduate Scholar.

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