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High molecular weight forms of basic fibroblast growth factor recognized by a new anti-bFGF antibody

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An antibody against basic fibroblast growth factor (bFGF) was raised using purified bovine pituitary bFGF. Western blot analysis revealed immunoreactive bands at 18, 24, 30–33 and 46 kDa in immunoaffinity purified extracts of pituitary and adrenal gland using this antibody. A similar staining pattern was obtained with ovary extracts with the exception of the missing 18 kDa band. A second anti-bFGF antibody raised against a synthetic peptide comprising the 24 N-terminal amino acids of bFGF reacted with the 18 kDa and the 46 kDa band of immunoaffinity purified ovary and adrenal gland extracts.

Fibroblast growth factor, basic; Ovary; Adrenal; Pituitary; Antibody

1. INTRODUCTION

Basic fibroblast growth factor (bFGF) is an angiogenic factor and a mitogen for many mesoderm-derived cells [1]. In addition, bFGF also influences proliferation and differentiation of glial cells [2,3]. Recently, in vivo and in vitro studies have shown that bFGF exerts neurotrophic actions in the peripheral (PNS) and central nervous system (CNS) [4–9]. Basic FGF has been purified from various tissues including brain, pituitary, corpus luteum, placenta, adrenal gland, kidney, and thymus (for reviews see [1,10]).

To further investigate the physiological role of bFGF immunological studies using antibodies against bFGF are a useful tool to elucidate: (i) the cellular and subcellular localization of bFGF in tissues from which bFGF can be isolated [11,12]; (ii) the developmental expression of bFGF in the CNS and PNS; (iii) the presence of bFGF in various tissues, cells and extracts with neurotrophic activity [12,13].

Basic FGF is a polypeptide of 146 residues. Tissue- and species-specific forms were found to originate from N-terminal truncation due to proteolysis that results in molecular masses between 16 and 18 kDa (for review see [10]). So far, higher molecular mass forms of bFGF have been isolated from guinea pig brain (25 kDa) [14] and rat brain (22 kDa) [15]. Immunological studies of Presta and co-workers [16] revealed immunoreactive bFGF-like proteins of 27 and 29 kDa in addition to the 18 kDa bFGF. The biological function of these high molecular weight forms remains to be resolved.

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Here we describe a polyclonal antibody against the bovine pituitary bFGF, which, in addition to the described immunoreactive bFGF-like proteins [16], recognizes a 46 kDa protein with putative neurotrophic activity.

2. MATERIALS AND METHODS

2.1. *Anti-bFGF antiserum*

New Zealand white rabbits were immunized with 130 μg aliquots of purified bovine pituitary bFGF (generous gift of D. Gospodarowicz). Two subcutaneous injections (1st week in Freund's complete adjuvant, 3rd week in incomplete adjuvant) were followed by 3 intravenous injections (4th, 5th and 10th week). Antibody titers were determined from enzyme-linked immunosorbent assay (ELISA) [17]. 96-well microtiter plates were coated with bFGF (20 ng/well), reacted with the antiserum, peroxidase-conjugated goat anti-rabbit IgG (Sigma) and *o*-phenylendiamine as a chromogen. IgG-fractions were purified by ammonium sulfate precipitation [18]. This antibody will be referred to as anti-$bFGF_{bp}$. An antibody (generous gift of P. Böhlen and A. Baird) against a synthetic peptide comprising the 24 N-terminal amino acids of bFGF (anti-$bFGF_{N\text{-}24}$) was also used.

2.2 *Affinity chromatography and Western blot analysis*

For enrichment of bFGF, bovine tissues (adrenal gland, ovary and pituitary) were extracted with 150 mM ammonium sulfate. Proteins precipitating between 1.9 and 3.8 M ammonium sulfate [19] were loaded onto an affinity column with anti-bovine bFGF IgG covalently bound to CNBr-activated Sepharose [20]. After elution of bound material with 2 M NaCl in phosphate buffer (10 mM, pH 7) or distilled water followed by NaCl/phosphate buffer, proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel-electrophoresis (PAGE) [21]. Following electrophoretic transfer onto nitrocellulose or immobilon (Millipore), anti-bFGF immunoreactive bands were visualized using the peroxidase-antiperoxidase method and diaminobenzidine as a chromogen. Control Western blots were developed without firstly using anti-bFGF antibody. In most cases immunoblotting on immobilon gave better results compared to nitrocellulose in terms of background staining and sharpness of bands.

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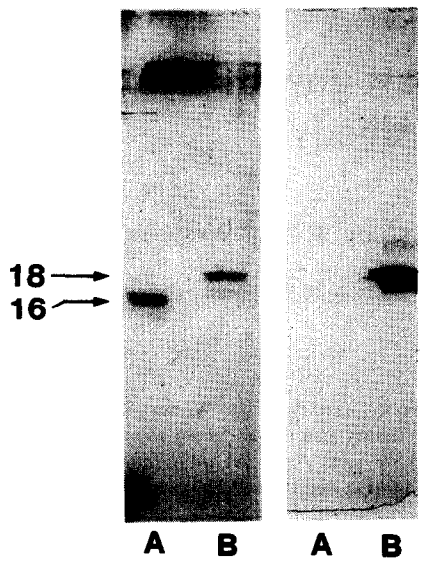

Fig.1. Silver stain of a 15% SDS-PAGE gel (left side) and Western blot analysis (right side) of acidic (A; 1.5 μg) and basic FGF (B; 1 μg) probed with anti-bFGF$_{bp}$ antibody.

2.3. *Bioassay*

Neurotrophic activity of bFGF-enriched fractions was tested in a bioassay using chick ciliary ganglionic neurons of embryonic day 8 (cCG 8) [6]. To establish plateau survival values we used saturating concentrations of purified or recombinant bovine bFGF [6] (recombinant bFGF was kindly provided by PROGEN, Heidelberg) or CNTF-containing cell-free extracts from selected embryonic chick eye tissues (CIPE) [22]. Culture medium without trophic factors served as the baseline control.

3. RESULTS

3.1. *Characterization of the anti-bFGF$_{bp}$ and antigens*

We first established that the antiserum against bFGF$_{bp}$ was specific in that it did not crossreact with acidic FGF (fig.1), a protein comprising about 50% sequence homology to bFGF [10]. Antibody titer (1:400, fig.2) as well as the detection limit (5–10 ng bFGF at a 1:50 dilution, fig.3) were obtained from ELISAs. Western blot analysis of several crude tissue extracts with anti-bFGF$_{bp}$ antiserum revealed no reactivity at all, or very weak reactivity with a 46 kDa polypeptide.

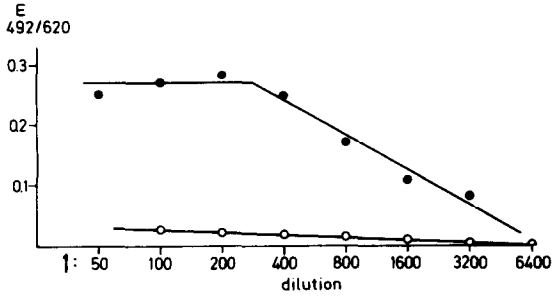

Fig.2. ELISA of anti-bFGF$_{bp}$ antibody (closed circles) and preimmune serum (open circles) on bFGF-coated microtiter plates (20 μg/well).

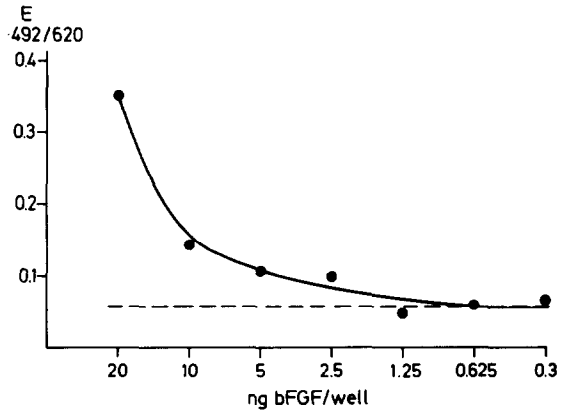

Fig.3. ELISA of anti-bFGF$_{bp}$ antibody (1:50 diluted) on bFGF-coated or control (dashed line; without antigen or coated with 2% BSA in PBS) microtiter plates.

Thus, for all further experiments extracts enriched in bFGF by ammonium sulfate precipitation [19] and anti-bFGF$_{bp}$ affinity chromatography were used. In such extracts from bovine pituitary and adrenal gland, besides the previously reported 18 kDa bFGF, further immunoreactive bands of about 24, 30–33 and 46 kDa were observed (fig.4). A similar staining pattern was obtained with ovary extracts; however, the 18 kDa band was virtually absent (fig.4).

We also subjected the bFGF-enriched extracts to Western blot analysis with anti-bFGF$_{N\text{-}24}$. This antibody also detected both, the 18 kDa and the 46 kDa band in bovine adrenal gland (fig.4) as well as ovary (fig.5).

3.2. *Neurotrophic activity of affinity-enriched bFGF fractions*

Fractions from bovine ovary containing the 18 as

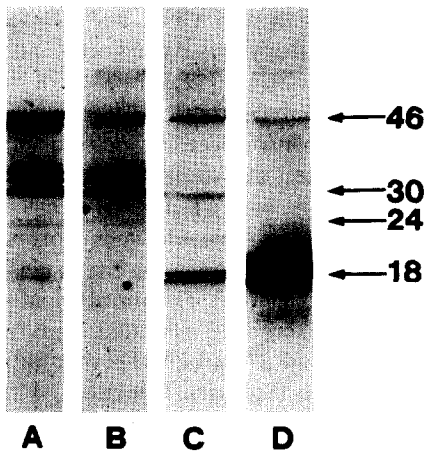

Fig.4. Western blot analysis of immunoaffinity purified extracts of pituitary (A; 50 μg), ovary (B; 50 μg), and adrenal gland (C and D; 100 μg) probed with anti-bFGF$_{bp}$ (A, B, C) and anti-bFGF$_{N\text{-}24}$ antibody (D), respectively.

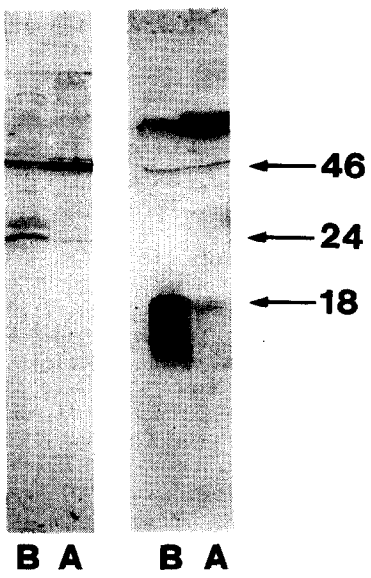

Fig.5. Western blot analysis of immunoaffinity purified extracts of ovary probed with anti-$bFGF_{bp}$ (left side) and anti-$bFGF_{N-24}$ (right side): (A) distilled water peak (100 μg); (B) NaCl peak (100 μg).

well as the 46 kDa immunoreactive bands were found to promote neuronal survival. Using a two-step elution from the anti-$bFGF_{bp}$ column with distilled water as the first and NaCl as the second eluant, we were able to separate the 46 kDa (water peak) from the 18 kDa (NaCl peak) band (fig.5). Both eluants exhibited comparable neurotrophic activities (ED_{50} of 46 kDa fraction = 0.8 μg/ml: ED_{50} of kDa fraction = 1.8 μg/ml) (fig.6).

4. DISCUSSION

We have demonstrated here that, in addition to the 18 kDa bFGF, higher molecular weigth forms exist in bovine tissues which are immunologically related to bFGF. Heparin-binding growth factors (HBGF) with molecular masses of 16–18 kDa have been isolated from a variety of tissues. These proteins seem to be closely related or identical to bFGF (for review see [10]). It is conceivable that the sequences of HBGFs, including those of higher molecular weights, contain conserved heparin-binding and receptor-binding domains but may differ with respect to extensions at the N-terminus and/or glycosylation [10]. In addition, studies of cDNA clones of bFGF showing an open reading frame extending beyond the 5′ end suggested that bFGF may be synthesized initially as a precursor [23] and that different bFGF forms might derive from processing of a precursor protein. Two high molecular mass forms of bFGF have been isolated so far which exist in addition to the 18 kDa bFGF in the respective tissues [14,15].

At present, 3 different antisera against bFGF (fig.3 in [16] and this study) have been characterized that recognize immunoreactive bFGF bands of a molecular mass of about 46 kDa. The finding that 18 kDa bFGF in ovary extracts is detected by the anti-$bFGF_{N-24}$, but not by the anti-$bFGF_{bp}$ antiserum, might reflect the presence of an ovary-specific 18 kDa bFGF whose domains for the latter antiserum are absent or masked.

The relatively high protein concentrations necessary for detection of neurotrophic activities could result from a loss of biological activity after immunoaffinity

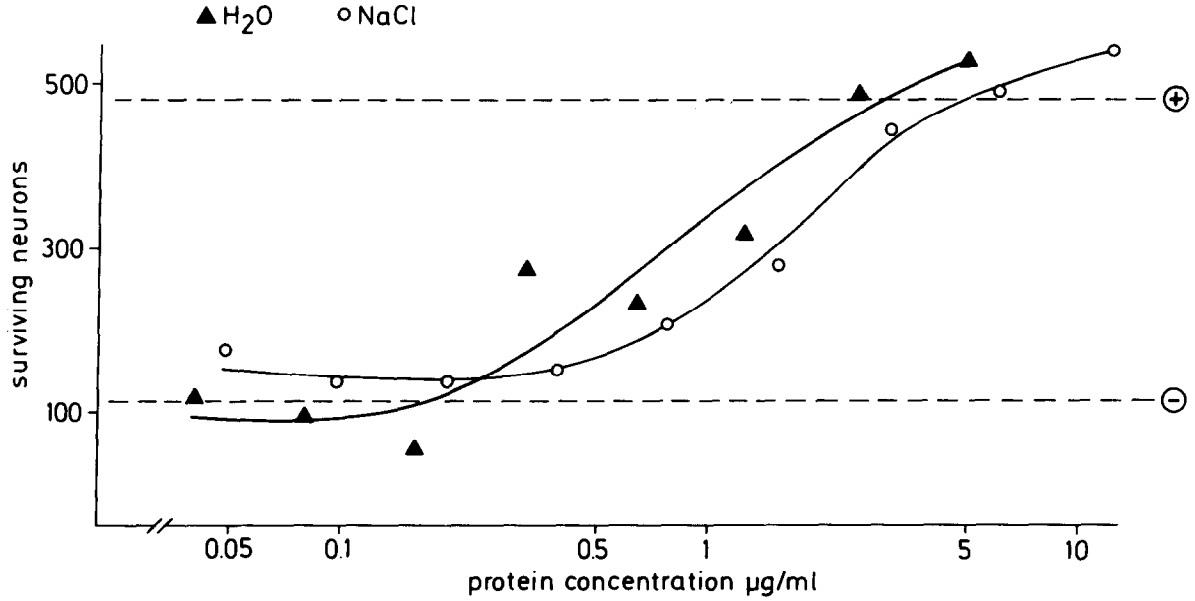

Fig.6. Representative dose-response curves on cCG 8 neurons of water peak and NaCl peak after immunoaffinity chromatography of ovary extracts.

enrichment or from impurities. However, cell blot analysis of medullary extract enriched for bFGF in the same way revealed neurotrophic activities at the respective molecular weights [12].

We conclude that besides the typical 16–18 kDa bFGF several tissues may additionally contain higher molecular weight forms of bFGF with putative biological activity.

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REFERENCES

[1] Gospodarowicz, D., Neufeld, G. and Schweigerer, L. (1986) Cell Diff. 19, 1–17.
[2] Gensburger, C., Labourdette, G. and Sensenbrenner, M. (1987) FEBS Lett. 217, 1–5.
[3] Weibel, M., Pettmann, B., Labourdette, G., Miehe, M., Bock, E. and Sensenbrenner, M. (1985) Int. J. Dev. Neurosci. 3, 617–630.
[4] Morrison, R.S., Sharma, A., de Vellis, J. and Bradshaw, R.A. (1986) Proc. Natl. Acad. Sci. USA 83, 7537–7541.
[5] Walicke, P., Cowan, W.M., Ueno, N., Baird, A. and Guillemin, R. (1986) Proc. Natl. Acad. Sci. USA 83, 3012–3016.
[6] Unsicker, K., Reichert-Preibsch, H., Schmidt, R., Pettmann, B., Labourdette, G. and Sensenbrenner, M. (1987) Proc. Natl. Acad. Sci. USA 84, 5459–5463.
[7] Anderson, K.J., Dam, D., Lee, A. and Cotman, C.W. (1988) Nature 332, 360–361.
[8] Otto, D., Unsicker, K. and Grothe, C. (1987) Neurosci. Lett. 83, 156–160.
[9] Grothe, C., Otto, D. and Unsicker, K. (1989) Neuroscience 31, 649–661.
[10] Lobb, R.R. (1988) Eur. J. Clin. Invest. 18, 321–336.
[11] Grothe, C. and Unsicker, K. (1989) J. Histochem. Cytochem. in press.
[12] Westermann, R., Johannsen, M., Unsicker, K. and Grothe, C. (1989) J. Neurochem. in press.
[13] Blottner, D., Westermann, R., Grothe, C., Böhlen, P. and Unsicker, K. (1989) Eur. J. Neurosci. 1, 471–478.
[14] Moscatelli, D., Joseph-Silverstein, J., Manejias, R. and Rifkin, D.B. (1987) Proc. Natl. Acad. Sci. USA 84, 5778–5782.
[15] Presta, M., Rusnati, M., Maier, J.A.M. and Ragnotti, G. (1988) Biochem. Biophys. Res. Commun. 155, 1161–1172.
[16] Presta, M., Foiani, M., Rusnati, M., Joseph-Silverstein, J., Maier, J.A.M. and Ragnotti, G. (1988) Neurosci. Lett. 90, 308–313.
[17] Engvall, E. and Perlman, P. (1972) J. Immunol. 109, 129–135.
[18] Harboe, N.M.G. and Inglid, A. (1973) Scand. J. Immunol. 2, Suppl. 1161–1164.
[19] Gospodarowicz, D., Massoglia, S., Cheng, J., Lui, G.M. and Böhlen, P. (1985) J. Cell. Physiol. 122, 323–332.
[20] Corfman, R.S. and Reeck, G.R. (1982) Biochim. Biophys. Acta 715, 170–174.
[21] Laemmli, U.K. (1970) Nature 227, 680–685.
[22] Barbin, G., Manthorpe, M. and Varon, S. (1984) J. Neurochem. 43, 1468–1478.
[23] Abraham, J.A., Mergia, A., Whang, J.I., Tumolo, A., Friedman, J., Hjerrild, K.A., Gospodarowicz, D. and Fiddes, J.C. (1986) Science 233, 545–548.