Retinoic Acid Receptor Belongs to a Subclass of Nuclear Receptors That Do Not Form "Docking" Complexes with hsp90†

Friedrich C. Dalman,‡ Laurie J. Sturzenbecker,§ Arthur A. Levin,§ Debra A. Lucas,§ Gary H. Perdew,∥ Martin Petkovitch,⊥ Pierre Chambon,⊥ Joseph F. Grippo,§ and William B. Pratt*,‡

Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, Michigan 48109, Department of Toxicology and Pathology, Hoffmann-La Roche Inc., Nutley, New Jersey 07110, Department of Foods and Nutrition, Purdue University, West Lafayette, Indiana 47907, and Laboratoire de Génétique Moléculaire et de Génie Génétique de l'INSERM, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg Cédex, France

Received January 24, 1991; Revised Manuscript Received March 7, 1991

ABSTRACT: We have recently reported that, in contrast to the glucocorticoid receptor, the thyroid hormone receptor does not bind to hsp90 when the receptor is translated in rabbit reticulocyte lysate [Dalman, F. C., Koenig, R. J., Perdew, G. H., Massa, E., & Pratt, W. B. (1990) *J. Biol. Chem. 265*, 3615–3618]. All of the steroid receptors that are known to bind hsp90 are recovered in the cytosolic fraction when hormone-free cells are ruptured in hypotonic buffer. In contrast, unliganded thyroid hormone receptors and retinoic acid receptors are tightly associated with nuclear components. In this paper, we translated the human estrogen receptor and the human retinoic acid receptor in reticulocyte lysate and then immunoadsorbed the [^{35}S]-methionine-labeled translation products with the 8D3 monoclonal antibody against hsp90. The estrogen receptor is bound to hsp90, as indicated by coimmunoadsorption, but the retinoic acid receptor is not. Translation and immunoadsorption of chimeric proteins containing the DNA binding domain of one receptor and the N-terminal and COOH-terminal segments of the other show that the DNA binding finger region of the estrogen receptor is neither necessary nor sufficient for hsp90 binding. These observations suggest that there are two classes within the steroid receptor family. In one class (e.g., glucocorticoid, mineralocorticoid, sex hormone, and dioxin receptors), the receptors bind to hsp90 and remain in some kind of inactive "docking" mode until hormone-triggered release of hsp90 occurs. In the retinoic acid/thyroid hormone class, the unligated receptors do not bind to hsp90, and the receptors appear to proceed directly to their high-affinity nuclear acceptor sites without entering the "docking" state.

The steroid–thyroid hormone superfamily of receptors (Evans, 1988) can be divided into two general subclasses according to the partitioning of the unliganded receptor in the cell. The first class of receptors, which is recovered in the cytosolic fraction after hypotonic cell rupture, includes the glucocorticoid, mineralocorticoid, progesterone, estrogen, androgen, and dioxin receptors (Pratt, 1987). Receptors in this group bind to hsp90 (Joab et al., 1984; Catelli et al., 1985; Sullivan et al., 1985; Schuh et al., 1985; Sanchez et al., 1985; Perdew, 1988) and remain in some kind of inactive "docking" complex, either in the cytoplasm (glucocorticoid receptor) or in loose association with the nucleus (e.g., estrogen and progesterone receptors), until binding of hormone triggers progression of the receptor to a high-affinity association with nuclear components where the primary events involved in transcriptional activation presumably occur.

In contrast to these receptors, the thyroid hormone receptor becomes tightly associated with the nucleus in hormone-free cells, and in its unliganded state, the thyroid hormone receptor can in some instances act as a transcriptional inhibitor (Koenig et al., 1989). Buffers containing high concentrations of salt are required to extract the unliganded thyroid hormone receptor from nuclear binding sites (Samuels et al., 1974; Spindler et al., 1975; Casanova et al., 1984). We recently reported that thyroid hormone receptors translated in a reticulocyte lysate system are not associated with hsp90 (Dalman et al., 1990). This stands in contrast to the in vitro translated glucocorticoid receptor which binds to hsp90 at the termination of translation (Dalman et al., 1989). These observations led to the proposal that binding of an unliganded receptor to hsp90 determines its retention in a docking complex that is recovered in the cytosolic fraction on cell rupture.

To test if this proposal applies in a more general manner, we have examined hsp90 binding by the retinoic acid receptor. It has recently been shown that the retinoic acid receptor is tightly associated with the nucleus of the hormone-free cell and high salt is required for its extraction from nuclear binding sites (Nervi et al., 1989). In this paper, we have translated the human retinoic acid receptor, the human estrogen receptor, and chimeras of the two receptors in rabbit reticulocyte lysate. We demonstrate that the estrogen receptor is bound to hsp90 while the retinoic acid receptor is not, suggesting that there are two subclasses of nuclear receptors that behave differently in their nuclear association in a manner that is determined by their ability to bind to hsp90.

Materials and Methods

Materials

L-[^{35}S]Methionine (1100 Ci/mmol) was supplied by DuPont–New England Nuclear. The riboprobe transcription system, SP6 and T7 RNA polymerases, plasmid pGem4, and rabbit reticulocyte lysate were obtained from Promega. A

† This investigation was supported by NIH Grants CA28010 (to W.B.P.) and ES04869 (to G.H.P.).

* To whom correspondence should be addressed.
‡ The University of Michigan Medical School.
§ Hoffmann-La Roche, Inc.
∥ Purdue University.
⊥ Laboratoire de Génétique Moléculaire et de Génie Génétique de l'INSERM.

0006-2960/91/0430-5605$02.50/0

plasmid containing the complete coding sequence for the rat glucocorticoid receptor (pT3.1118) was kindly provided by Dr. Keith Yamamoto (Miesfeld et al., 1986), and plasmids for the human estrogen receptor (HEGO) and the α- and β-retinoic acid receptors were constructed as previously described (Tora et al., 1989; Brand et al., 1988). Construct A (ER-RAR-α.CAS) is a chimeric receptor in which the DNA binding cassette of the estrogen receptor (HE28) (Green & Chambon, 1987) was replaced with the corresponding region of the α-retinoic acid receptor. Thus, residues 185–250 were removed from HE28 and replaced with residues 88–153 of the α-retinoic acid receptor. In making this construct, the insertion of unique *Kpn*I and *Xho*I sites introduces two (Gly and Thr) and three (Ala, Arg, and Glu) amino acids as previously described (Green & Chambon, 1987). Construct B (RAR-β-ER.CAS) is a chimeric receptor in which the DNA binding cassette of the β-retinoic acid receptor was replaced with the corresponding region (residues 185–205) of the human estrogen receptor (HE28) (Green & Chambon, 1987) as described (Brand et al., 1988). The 8D3 IgM monoclonal antibody against hsp90 has been described previously (Perdew, 1988).

Methods

In Vitro Transcription and Translation. The cDNA clone for the glucocorticoid receptor was inserted into a pGem4 plasmid, linearized, and transcribed with SP6 polymerase. The cDNA clones for the retinoic acid, estrogen, chimeric receptors were cloned into the expression vector pSG5 (Green et al., 1988), linearized, and transcribed with T7 polymerase. Transcription and translation were carried out according to standard Promega protocols as described previously (Dalman et al., 1989). Following translation, translation products were diluted 5-fold into a solution of 10% glycerol, 1 mM EDTA, 10 mM Hepes, pH 7.4, and 5 mM DTT (HEGD) plus 20 mM molybdate. Aliquots (50 μL) of the diluted translation products were then boiled in 2 × SDS sample buffer, and one-fourth of each sample was subsequently analyzed by SDS–PAGE and autoradiography. Alternatively, translation products were assayed for association with hsp90 by using the following immunoadsorption protocol.

Immunoadsorption. Goat anti-mouse IgM was rotated at 4 °C with protein A–Sepharose in HEGD for 1 h. The Sepharose beads were then washed twice with 1-mL aliquots of HEGD and rotated for another hour with either 8D3 antibody or nonimmune IgM. The pellets were subsequently washed twice with 1-mL aliquots of HEGD, and once with 1 mL of HEGD plus 20 mM molybdate and 2.5% bovine serum albumin. For each immunoadsorption condition, the pellets consisted of 25 μL of ascites fluid and either 8D3 from 100 μL of ascites fluid or an equivalent amount of nonimmune IgM. To immunoadsorb translation products, the pellets were resuspended in 0.5 mL of HEGD plus molybdate and bovine serum albumin and mixed with 50-μL aliquots of 5-fold-diluted translation products. The suspension was rotated for 45 min at 4 °C, and pellets were washed 3 times with ice-cold HEGD plus molybdate. Immunoadsorbed proteins were eluted from the pellets by boiling in 2 × SDS sample buffer, and one-fourth of each sample was subsequently analyzed by SDS–PAGE and autoradiography.

Gel Electrophoresis and Autoradiography. SDS–polyacrylamide gel electrophoresis was performed in 10% slab gels as previously described (Dalman et al., 1989). Following electrophoresis, proteins were stained with Coomassie blue, the gels were incubated in Amplify and dried, and autoradiography was performed at −70 °C.

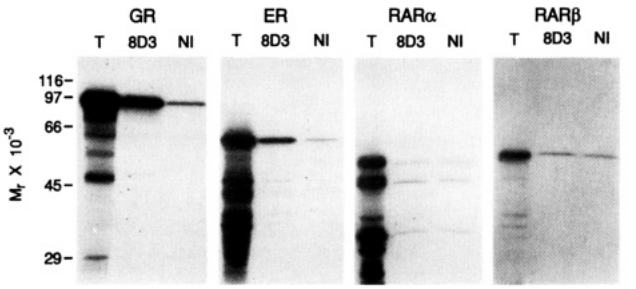

FIGURE 1: In contrast to the glucocorticoid (GR) and estrogen (ER) receptors, the α (RARα) and β (RARβ) forms of the retinoic acid receptor are not associated with hsp90 after translation in rabbit reticulocyte lysate. Aliquots of reticulocyte lysate containing [^{35}S]methionine-labeled receptor as indicated above each panel were immunoadsorbed by the 8D3 monoclonal antibody against hsp90 or by nonimmune IgM, and the immunoadsorbed protein was resolved by SDS–PAGE and autoradiography. In each panel, T denotes the lane that contains the total translation products before immunoabsorption, 8D3 denotes the lane containing the material immunoadsorbed by 8D3, and NI denotes the lane containing material immunoadsorbed by the nonimmune antibody.

Results and Discussion

To ask if the retinoic acid receptor associates with hsp90, we translated the α and β forms of the retinoic acid receptor in reticulocyte lysate and immunoadsorbed the [^{35}S]-methionine-labeled products with the 8D3 antibody. The 8D3 is a monoclonal IgM antibody that immunoadsorbs receptor–hsp90 complexes (Perdew, 1988). Figure 1 shows the immunoadsorption by 8D3 and nonimmune IgM of ^{35}S-labeled glucocorticoid and estrogen receptors as well as the α and β forms of the retinoic acid receptor following their translation in rabbit reticulocyte lysate. By comparing the 8D3 lane with the nonimmune (NI) lane in each panel, it is clear that the glucocorticoid and estrogen receptors are immune specifically absorbed whereas the retinoic acid receptors are not.

We have shown previously (Dalman et al., 1989) that nearly all of the glucocorticoid receptors can be immunoadsorbed after translation in vitro if a high enough concentration of 8D3 antibody is added to the sample. In order to conserve antibody in this work, we have immunoadsorbed 50–60% of the total glucocorticoid receptor as determined by cutting out and counting the bands of full-length translation product from gels such as that of Figure 1. Thirty to forty percent of the full-length estrogen receptor is immunoadsorbed by 8D3 under the same conditions. The lower yield may reflect a weaker association of hsp90 with the newly translated estrogen receptor than with the glucocorticoid receptor. The absence of immune-specific absorption of the α- and β-retinoic acid receptors demonstrates that they do not form a stable complex with hsp90.

Both genetic (Pratt et al., 1988) and biochemical (Denis et al., 1988; Bresnick et al., 1989) studies have demonstrated that hsp90 binds to the hormone binding domain of the glucocorticoid receptor. In the case of the estrogen receptor, however, it has been reported (Baulieu et al., 1989) that a mutant receptor [HE4 in Kumar et al., (1987)] lacking the DNA binding domain does not form the 8S receptor–hsp90 complex. This has led to a model in which both the DNA binding zinc fingers and hormone binding domain of the estrogen receptor are critical for forming a stable complex with hsp90 (Baulieu et al., 1989).

The construction of chimeric proteins in which the DNA binding domain of the estrogen or retinoic acid receptor has been replaced with the DNA binding domain of the other (Kumar et al., 1987) provides a system in which to test this model. Accordingly, we translated the two chimeras diag-

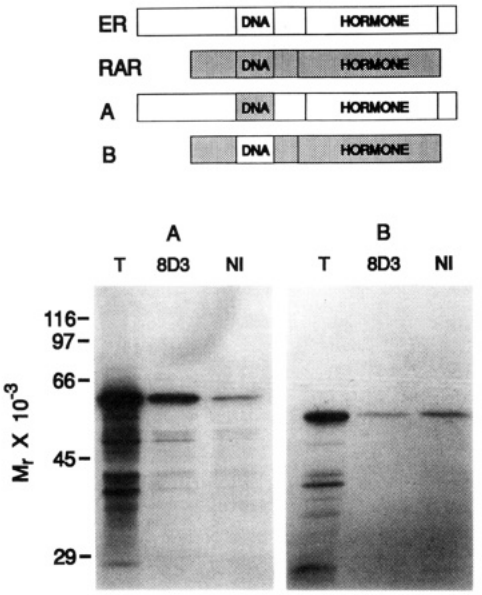

FIGURE 2: Zinc finger region of the estrogen receptor DNA binding domain is neither necessary nor sufficient for the estrogen receptor to bind hsp90. Aliquots of reticulocyte lysate containing [^{35}S]-methionine-labeled construct B were immunoadsorbed by 8D3 or by nonimmune antibody, and the immunoadsorbed protein was resolved by SDS–PAGE and autoradiography. Lanes in each panel are as noted in the legend to Figure 1. Above the two panels, the domain maps of the estrogen receptor, the retinoic acid receptor, and the chimeras A and B are presented. As the shading indicates, chimera A is an estrogen receptor containing the DNA binding domain of the retinoic acid receptor, and chimera B is a retinoic acid receptor containing the zinc finger region of the DNA binding domain of the estrogen receptor (see Materials for the details of each construction).

rammed in Figure 2 and immunoadsorbed them with the 8D3 antibody. It is clear that construct A (ER-RAR-α.CAS), which contains the hormone binding domain of the estrogen receptor and the DNA binding domain of the retinoic acid receptor, binds hsp90. In contrast, construct B (RAR-β-ER.CAS), which contains the hormone binding domain of the retinoic acid receptor and most of the DNA binding domain of the estrogen receptor, does not bind hsp90. The region of the DNA binding domain inserted into the retinoic acid receptor in construct B contains residues 185–250 of the human estrogen receptor, a region that spans both zinc fingers (Green & Chambon, 1988).

These results demonstrate that the zinc finger region of the estrogen receptor DNA binding domain is neither necessary nor sufficient for the estrogen receptor to bind hsp90. Construct B, however, does not contain a COOH-terminal segment of the DNA binding domain that extends from amino acid 251 to amino acid 271 (Kumar et al., 1987). The HE4 mutant estrogen receptor that yields predominantly 4S estrogen receptor (Chambraud et al., 1990) is deleted for amino acids 199–264 (Kumar et al., 1987), and it is therefore possible that some feature between amino acid 250 and the COOH-terminus of the estrogen receptor DNA binding domain is required for a stable receptor–hsp90 complex. This would be consistent with the data of Chambraud et al. (1990) implicating a feature between amino acids 251 and 271 in forming the 8–9S estrogen receptor complex.

The fact that the unliganded retinoic acid and thyroid hormone receptors both form tight complexes with nuclei that require high salt conditions for receptor extraction (Samuels et al., 1974; Spindler et al., 1975; Casanova et al., 1984; Nervi et al., 1989) differentiates those receptors from the larger class of steroid receptors which are able to form cytosolic "docking" complexes. We have now shown that both the retinoic acid receptors (Figure 1) and thyroid hormone receptors (Dalman et al., 1990) do not bind hsp90. This stands in contrast to the receptors that can form "docking" complexes, all of which bind hsp90. Taken together, these observations suggest that the weak versus tight nuclear binding characteristics of the unliganded receptors in the two subclasses may reflect their association with hsp90.

It has been proposed (Pratt et al., 1988) that a short (20 amino acid) region of the hormone binding domain that is highly conserved through all of the steroid–thyroid hormone family of receptors is the binding site for hsp90. We have recently shown, however, that mouse glucocorticoid receptor that is deleted for this region still binds hsp90 (Housley et al., 1990). Thus, the major determinants for hsp90 binding must lie outside this region. The fact that hsp90 does not bind to retinoic acid and thyroid hormone receptors will be useful in defining the critical features required for hsp90 binding. Obviously, the structures required for hsp90 binding should be conserved in the glucocorticoid, mineralocorticoid, sex hormone, and dioxin receptors, but they should not be conserved in thyroid hormone and retinoic acid receptors.

Registry No. Retinoic acid, 302-79-4.

References

Baulieu, E. E., Binart, N., Cadepond, F., Catelli, M. G., Chambraud, B., Garnier, J., Gasc, J. M., Groyer-Schweizer, G., Oblin, M. E., Radanyi, G., Redeuilh, G., Renoir, J. M., & Sabbah, M. (1989) in *The Steroid/Thyroid Hormone Receptor Family and Gene Regulation* (Carlstedt-Duke, J., Eriksson, H., & Gustafsson, J. A., Eds.) pp 301–318, Birkhäuser Verlag, Basel, Switzerland.

Brand, N., Petkovitch, M., Krust, A., Chambon, P., de The, H., Marchio, A., Tiollais, P., & Dejean, A. (1988) *Nature 322*, 850–853.

Bresnick, E. H., Dalman, F. C., Sanchez, E. R., & Pratt, W. B. (1989) *J. Biol. Chem. 264*, 4992–4997.

Casanova, J., Horowitz, Z. D., Copp, R. P., McIntyre, W. R., Pascual, A., & Samuels, H. H. (1984) *J. Biol. Chem. 259*, 12084–12091.

Catelli, M. G., Binart, N., Jung-Testas, I., Renoir, J. M., Baulieu, E. E., Feramisco, J. R., & Welch, W. J. (1985) *EMBO J. 4*, 3131–3135.

Chambraud, B., Berry, M., Redeuilh, G., Chambon, P., & Baulieu, E. E. (1990) *J. Biol. Chem. 265*, 20686–20691.

Dalman, F. C., Bresnick, E. H., Patel, P. D., Perdew, G. H., Watson, S. J., & Pratt, W. B. (1989) *J. Biol. Chem. 264*, 19815–19821.

Dalman, F. C., Koenig, R. J., Perdew, G. H., Massa, E., & Pratt, W. B. (1990) *J. Biol. Chem. 265*, 3615–3618.

Denis, M., Gustafsson, J. A., & Wikstrom, A. C. (1988) *J. Biol. Chem. 263*, 18520–18523.

Evans, R. M. (1988) *Science 240*, 889–895.

Green, S., & Chambon, P. (1987) *Nature 325*, 75–78.

Green, S., & Chambon, P. (1988) *Trends Genet. 4*, 309–314.

Green, S., Issemann, I., & Scheer, E. (1988) *Nucleic Acids Res. 16*, 369.

Housley, P. R., Sanchez, E. R., Danielsen, M., Ringold, G. M., & Pratt, W. B. (1990) *J. Biol. Chem. 265*, 12778–12781.

Joab, I., Radanyi, C., Renoir, J. M., Buchou, T., Catelli, M. G., Binart, N., Mester, J., & Baulieu, E. E. (1984) *Nature 308*, 850–853.

Koenig, R. J., Lazar, M. A., Hodin, R. A., Brent, G. A., Larson, P. R., Chin, W. W., & Moore, D. D. (1989) *Nature 337*, 659–661.

Kumar, V., Green, S., Stack, G., Berry, M., Jin, J. R., & Chambon, P. (1987) *Cell 51*, 941-951.
Miesfeld, R., Rusconi, S., Godowski, P. J., Maler, B. A., Okret, S., Wikstrom, A. C., Gustafsson, J. A., & Yamamoto, K. R. (1986) *Cell 46*, 389-399.
Nervi, C., Grippo, J. F., Sherman, M. I., George, M. D., & Jetten, A. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5854-5858.
Perdew, G. H. (1988) *J. Biol. Chem. 263*, 13802-13805.
Pratt, W. B. (1987) *J. Cell. Biochem. 35*, 51-68.
Pratt, W. B., Jolly, D. J., Pratt, D. V., Hollenberg, S. M., Giguere, V., Cadepond, F. M., Schweizer-Groyer, G., Catelli, M. G., Evans, R. M., & Baulieu, E. E. (1988) *J. Biol. Chem. 263*, 267-293.
Samuels, H. H., Tsai, J. S., Casanova, J., & Stanley, F. (1974) *J. Clin. Invest. 54*, 853-865.
Sanchez, E. R., Toft, D. O., Schlesinger, M. J., & Pratt, W. B. (1985) *J. Biol. Chem. 260*, 12398-12401.
Schuh, S. W., Yonemoto, J., Brugge, V. J., Bauer, R. M., Riehl, W. P., Sullivan, W. P., & Toft, D. O. (1985) *J. Biol. Chem. 260*, 14292-14296.
Spindler, S. R., MacLeod, K. M., Ring, J., & Baxter, J. D. (1975) *J. Biol. Chem. 250*, 4113-4119.
Sullivan, W. P., Vroman, B. T., Bauer, V. J., Puri, R. K., Riehl, R. M., Pearson, G. R., & Toft, D. O. (1985) *Biochemistry 24*, 4214-4222.
Tora, L., Mullick, A., Metzger, D., Ponglikitmongkol, M., Park, I., & Chambon, P. (1989) *EMBO J. 8*, 1981-1986.

Mutations in the Heparin-Binding Domains of Human Basic Fibroblast Growth Factor Alter Its Biological Activity

William F. Heath,* Amanda S. Cantrell, Nancy G. Mayne, and S. Richard Jaskunas
Lilly Research Laboratories, Indianapolis, Indiana 46285
Received August 6, 1990; Revised Manuscript Received February 25, 1991

ABSTRACT: Eleven structural analogues of human basic fibroblast growth factor (bFGF) have been prepared by site-directed mutagenesis of a synthetic bFGF gene to examine the effect of amino acid substitutions in the three putative heparin-binding domains on FGF's biological activity. After expression in Escherichia coli, the mutant proteins were purified to homogeneity by use of heparin–Sepharose chromatography and analyzed for their ability to stimulate DNA synthesis in human foreskin fibroblasts. Recombinant human bFGF 1-146 and [Ala^{69},Ser^{87}]bFGF, an analogue where two of the four cysteines had been replaced by alanine and serine, were equipotent to standard bovine basic fibroblast growth factor. Substitution of aspartic acid-19 by arginine in the first heparin-binding domain yielded a molecule that stimulated a higher total mitogenic response in fibroblasts as compared to bFGF. In addition, replacement of either arginine-107 in the second domain or glutamine-123 in the third domain with glutamic acid resulted in compounds that were 2 and 4 times more potent than bFGF. In contrast, substitution of arginine-107 with isoleucine reduced the activity of the molecule by 100-fold. Combination of domain substitutions to generate the [Glu^{107,123}]bFGF and [Arg^{19},Lys^{123,126}]bFGF mutants did not show any additivity of the mutations on biological activity. Alterations in the biological activity of the analogues was dependent on both the site of and the type of modification. Increased positive charge in the first domain and increased negative charge in the second and third domains enhanced biological potency. The altered activities of the derivatives appear to be due in part to changes in the affinity of the analogues for heparin. We conclude that changes in all three of the putative heparin–binding domains result in altered mitogenic activity and heparin interaction of basic fibroblast growth factor.

Basic fibroblast growth factor is a member of a family of heparin-binding polypeptide growth factors that have been shown to be potent mitogens for a wide variety of cells of mesodermal and neuroectodermal origin (Gospodarowicz et al., 1987). In addition, basic FGF[1] has been shown to be a potent stimulator of angiogenesis in vivo (Gospodarowicz et al., 1979, 1984; Esch et al., 1985) and to stimulate collagenase and plasminogen activator secretion, chemotaxis, and mitogenesis in capillary endothelial cells in vitro (Presta et al., 1986). The discovery of the strong affinity of FGF for heparin (Shing et al., 1984) has allowed the isolation and characterization of a number of endothelial cell mitogens of 13 000–18 000 molecular weight with a strong affinity for heparin and a basic isoelectric point (p*I*) (Lobb et al., 1986). Further work has shown that these factors are all forms of basic FGF differing in their N-terminal sequences as a result of cleavage by acid proteases (Klagsbrun et al., 1987). The significance of this amino-terminal heterogeneity is not known. Pituitary-derived basic fibroblast growth factor has been shown to be a single chain protein consisting of 146 amino acids (Esch

* To whom correspondence should be addressed at Lilly Research Laboratories, MC797, 88/4, Indianapolis, IN 46285.

[1] Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; DME, Dulbecco's-modified Eagle's medium; ED_{50}, half-maximal stimulatory dose; FBS, fetal bovine serum; FGF, fibroblast growth factor; rbFGF, recombinant Met-Arg-Leu-[Ala^{69},Ser^{87}]bFGF; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0006-2960/91/0430-5608$02.50/0