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Mapping a Heparin Binding Site on ErbB-3 Epidermal Growth Factor Receptor

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Signaling via the ErbB-family of receptors plays an important role in mammalian development and oncogenesis. Here we show that the ErbB-3 receptor, but not other members of this receptor family, binds to immobilized heparin and can be dissociated only at a high ionic strength comparable to that required for fibroblast growth factor receptors. Competition-binding analysis suggests that this interaction is specific and requires highly sulfated species of heparan sulfate. Primary sequence analysis of ErbB-3 identified a basic amino acid cluster ^{466}KHNRPRR^{472} localized to the proximal, cysteine-rich extracellular ligand binding domain of the receptor, with charge density and distribution compatible with, but different to, known linear heparin binding motifs. Site-directed mutagenesis, replacing this sequence with the corresponding residues from ErbB-1, resulted in complete loss of heparin binding activity of the chimeric receptor. Finally, antibodies directed to the putative heparin binding peptide, efficiently bind the native receptor suggesting a novel target for blocking heparin mediated ErbB-3 interactions.

The ErbB3 family of receptor tyrosine kinases plays an important role in the regulation of major biological processes, affecting cell proliferation, differentiation and morphogenesis (1, 2). Two members of the ErbB family, ErbB-3, and ErbB-4 interact with high affinity with other members of the ErbB family, such as ErbB-1 (the EGF receptor) and ErbB-2, thus serving as essential coreceptors by forming heterodimers which are indispensable for their signal transduction and oncogenic potential. ErbB-3 (3–6), which has impaired tyrosine kinase activity (2) in particular may serve as a ubiquitous coreceptor and a signal amplifier by joining a kinase competent receptor such as ErbB-1 or ErbB-2.

Direct heparin binding activity of receptor tyrosine kinases was first suggested for FGF1 and 2 (7, 8) and for FGF receptor 1 (9) where a point mutation in its putative heparin binding domain was sufficient to abolish heparin binding and heparin-dependent receptor activation. The requirement for heparan sulfates for multiple FGF ligands and receptors and the details of their interaction were extensively investigated (10–18) as well as the identification of other growth factors, such as the vascular endothelial growth factor (VEGF) (19–21) and hepatocyte growth factor (22) as heparin binding systems which require heparan sulfates for optimal biological activity. High affinity binding of HB-EGF to, and phosphorylation of ErbB-1 seems also to require heparin, despite no detectable affinity of the ErbB-1 receptor to heparin (23).

Classical heparin binding motifs on proteins have been identified and described based on sequence organization of their basic and nonbasic residues (24) and consensus sequences for glycosaminoglycan recognition were determined as [XBBXBX], [XBBBXXBX], and later as [XBBXXBBBXXBBX] (25), where B is the probability of a basic residue and X is a hydrophathic residue. While there is general agreement that HS-protein interactions are primarily electrostatic by nature, there is increasing evidence that these interactions may involve multiple, ionic and nonionic interactions localized within specific protein modules and structural motifs. As ErbB-3 interacts primarily with heparin binding ligands, such as the neuregulins, we sought to test whether it can directly bind heparin and if so, to characterize potential structural elements in its extracellular domain, which may promote this activity. We find a novel heparin binding consensus sequence on ErbB3 sharing a prototypic regulatory HS binding motif which may be directly involved in regulating cell surface ligand–receptor interactions.

MATERIALS AND METHODS

Site-directed mutagenesis and construction of a soluble heparin binding mutant of ErbB-3. The putative heparin-binding domain of ErbB-3 located at amino acids 466–472 (KHNRPRR) was replaced with the homologous region from ErbB-1 (EGFR) [amino acids 467–473 (ISNRGEN)] that totally lacks heparin-binding ability. Site di-

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rected mutagenesis was performed according to Higuchi *et al.*, 1988. In brief, first PCR was performed using pairs of primers containing the mismatched oligonucleotide and the corresponding 5′ or 3′ primer. The primer at the 5′ was GAGATCACAGGTTACCTGAAC located at position 1372–1392 of the ErbB-3 cDNA and the primer at the 3′ was CCCTCAGGGATCCACACTCC located at position 2374–2390. Human ErbB-3 cDNA served as a template. After completion of the primary PCRs, aliquots were size fractionated and isolated on 1% of low melting agarose gel that served as target DNA for the secondary PCR with the 5′ and 3′ above primers. The final mutated PCR product (~1 kb) was cloned into a pGEM-T vector (Promega, Madison, WI), sequenced, excised from the vector using *Bst*EII–*Bam*HI sites and cloned into the ErbB-3 cDNA which was digested with the same restriction enzymes.

For construction of the soluble ErbB-3 mutant the above 5′ primer and a reverse primer GAAGATCTGGTTTTGCCGATCAGCAACC at position 2100–2118 containing an additional *Bgl*II restriction site, were used. The ErbB-3 heparin-binding mutant (mut ErbB-3) cDNA served as a template. The resulting PCR product was digested with *Bst*EII–*Bgl*II and subcloned in place of the homologous region in the wild type ErbB-3 alkaline phosphatase expression vector (6).

Binding of soluble ErbB-3 and its heparin binding site mutant to heparin. Conditioned medium containing the extracellular part of both receptors expressed as either alkaline or human IgG Fc fusion protein in 293T cells was incubated with heparin-Sepharose beads for 18 h at 4°C. After excessive washing with PBS, the beads were washed three times with or without increasing concentrations of NaCl, heparin or heparin-derived fragments. All the samples were then washed twice with PBS and the bound receptor level was estimated according to the associated alkaline phosphatase activity determined by incubation with the phosphatase substrate *p*-nitrophenyl phosphate (Sigma 104) and reading the absorbance at 410 nm, in the case of AP fusion and by further incubation with horse radish peroxidase conjugated goat anti-human FC for the Ig-Fc fusion constructs, and reading the absorbance at 540 nm.

Generation and characterization of polyclonal antibodies to the putative heparin binding site of ErbB-3. A peptide having the sequence FERLDIKHNRPRRDC corresponding to the putative binding epitope of ErbB-3 was coupled to KLH using MBS (Pierce, Rockford, IL) according to manufacturers instructions and used for immunizing both rabbits and mice. Antiserum was tested for specific recognition of the wild type and mutant receptors by either SDS–PAGE and Western immunoblotting or by direct ELIZA of lysates from 293 T cells expressing either receptor.

RESULTS

To test the possibility that members of the ErbB family of receptor tyrosine kinases and particularly ErbB-3 may directly bind heparin-like molecules, we expressed all four ErbB receptor extracellular domains as soluble fusion proteins with either human placental alkaline phosphatase or human heavy chain Fc proteins, as previously described (6, 26). Each soluble receptor was then tested for its capacity to bind to immobilized heparin at physiological pH and varying ionic strength. As can be seen in Fig. 1A, under these conditions ErbB-3, but not any of the other members of this family, has a significant affinity to heparin, comparable in that sense to the well-known heparin binding, fibroblast growth factor receptors. These results suggest that the ErbB-3 receptor has a unique ability among the ErbB-family of receptors to directly bind heparin.

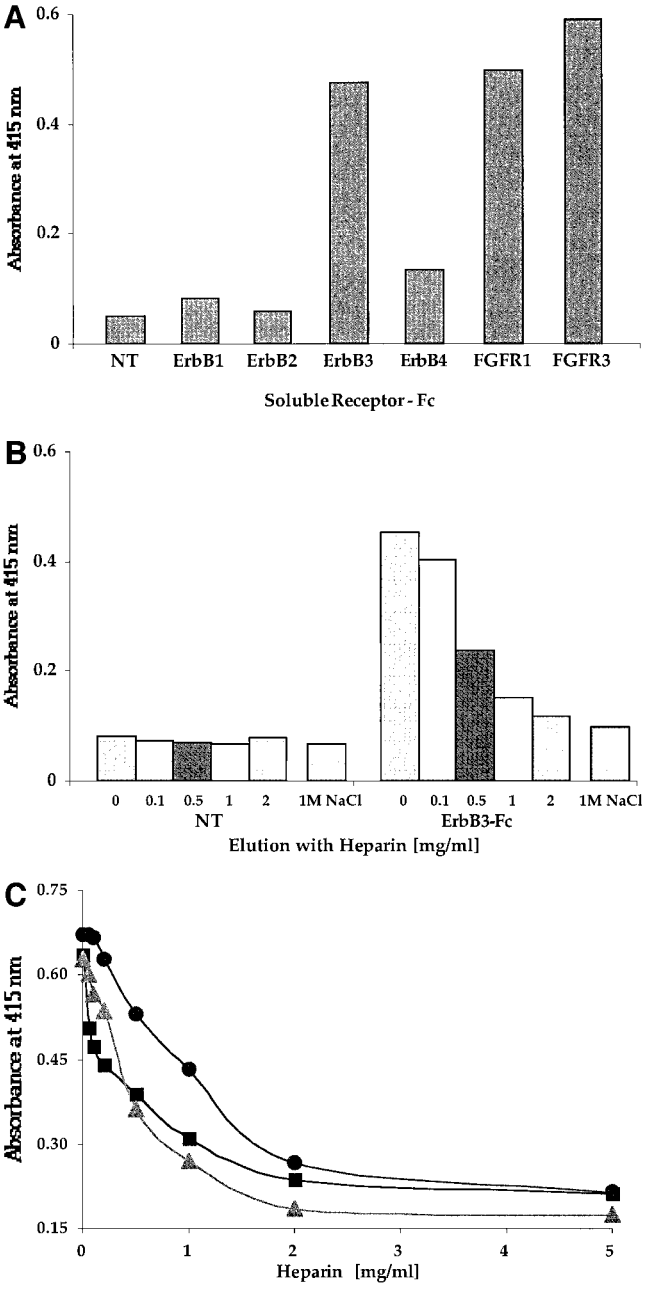


FIG. 1. Heparin binding of soluble ErbB receptor family members. (A) Heparin chromatography of soluble ErbB-Fc receptors was determined after extensive washing with PBS and compared with that of the known heparin binding FGF receptors 1 and 3. (B) Heparin affinity of conditioned medium from 293 T nontransfected or expressing ErbB-3 receptors was determined by elution with increasing concentrations of heparin compared with elution with 1 M of NaCl. (C) Specific association of soluble ErbB-3, FRFR1, and FGFR3 immobilized on heparin-sepharose beads was determined by competition with increasing concentrations of free heparin.

Binding of the ErbB-3-Fc produced in cells genetically engineered to express the soluble receptor, could be displaced with either free heparin or with high salt (Fig. 1B) suggesting a reversible, mostly ionic, interac-

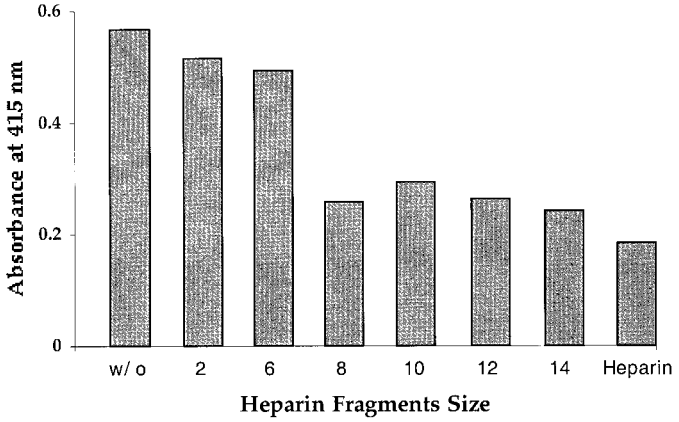


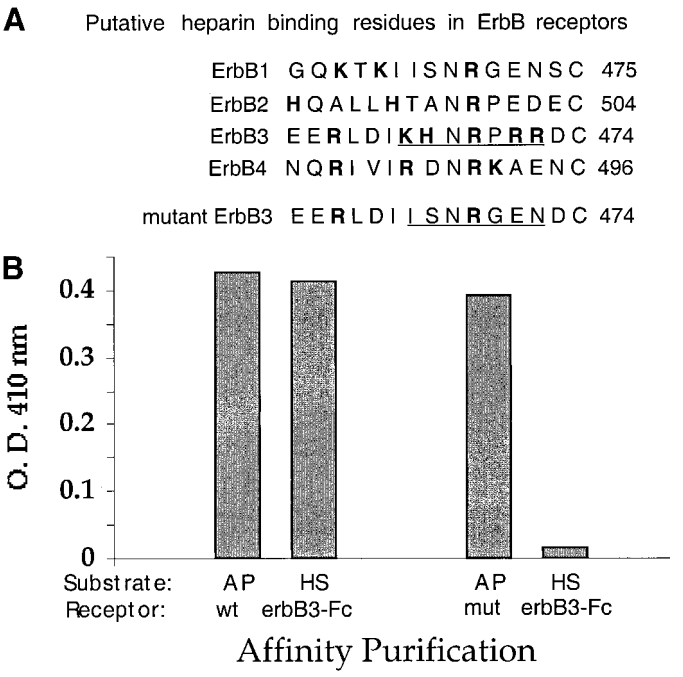
FIG. 2. Effect of heparin fragments on binding of soluble ErbB-3 to heparin. Heparin oligosaccharides of 2–14 saccharides in length were tested for their capacity to compete for specific binding of ErbB-3 to immobilized heparin. Crude heparin was used as a control.

tion of this receptor with heparin. No background binding was detected in the conditioned media of cells which were not engineered to express the fusion protein. This binding is specific and could be competed in a dose response manner by soluble heparin with half maximal competition at 200 μg/ml, similar to that required for FGFR3 and FGFR1 binding (Fig. 1C).

The minimal size of heparin fragments required to elute ErbB-3 soluble receptor was found by competition analysis to be of 8 monosaccharides in length (Fig. 2). There was no effect of a heparin hexasaccharide on this interaction, even at a higher molar ratio compared to the octasaccharides. Larger fragments seem to be equipotent to crude heparin in their overall capacity to compete for ErbB-3 heparin interaction. These results may imply a rather large heparin binding surface or region similar in that respect to that found on FGF receptors and significantly larger than that observed for FGF ligands, such as FGF2 (27, 28) which correspondingly can be efficiently displaced from immobilized heparin by heparin fragments as small as tetrasaccharides (23).

Primary sequence analysis based on consensus heparin binding (24, 25, 29, 30) identified a cluster of basic amino acids spaced in a fashion compatible with a linear putative heparin-binding site and located at the carboxy terminal end of the third sub-domain, comprising a putative link between the third and the fourth cysteine rich domains (Fig. 3A). In order to assess the role of this epitope in heparin binding, we replaced the region encoding amino acids 466–472 (KHNRPRR) in ErbB-3 with that of the homologous region in ErbB-1 (EGFR), encoding for amino acids 467–473 (ISNRGEN) (Fig. 3A). ErbB-1 totally lacks heparin-binding ability (Fig. 1A) and its activation by EGF is not dependent on heparin (23).

To determine the capacity of the mutant ErbB-3 versus the wild type receptor to bind heparin, the extracellular part of both receptors, expressed as alkaline phosphatase fusion protein in 293T cells was loaded onto heparin-Sepharose beads. Wild type ErbB-3 was retained on the column and as expected could be eluted only by high salt (0.4 M NaCl) (Fig. 3B). The mutant chimeric ErbB-3 receptor, however, did not bind to the



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FGFR1	A	P	Y	W	T	S	P	E	**K**	M	E	**K**	**K**	L	**H**	A	V	P
FGFR2	A	P	Y	W	T	N	T	E	**K**	M	E	**K**	**R**	L	**H**	A	V	P
FGFR3	A	P	Y	W	T	**R**	P	E	**R**	M	D	**K**	**K**	L	L	A	V	P
FGFR4	A	P	Y	W	T	**H**	P	Q	**R**	M	E	**K**	**K**	L	**H**	A	V	P
ErbB3	P	T	E	E	**R**	L	D	I	**K**	**H**	N	**R**	P	**R**	**R**	D	C	V
VEGFR1	S	N	I	S	T	P	**R**	P	V	**K**	L	L	**R**	G	**H**	T	L	V
VEGFR2	F	N	W	E	Y	P	S	S	**K**	**H**	Q	**H**	**K**	**K**	L	V	N	**R**
c-MET	**H**	P	L	**H**	S	I	S	V	**R**	**R**	L	**K**	E	T	**K**	D	G	F
IL-2Rα	T	M	L	N	C	E	C	**K**	**R**	G	F	**R**	**R**	I	**K**	S	G	S
M-CSF-R	A	G	V	S	L	V	**R**	V	**R**	G	**R**	P	L	M	**R**	**H**	T	N

FIG. 3. (A) Sequence alignment and mapping of putative regulatory HSB domains on ErbB3 and other heparin binding growth factor receptors. Mapping the heparin binding site of ErbB-3 by site directed mutagenesis. Sequence alignment of the ErbB receptor family searching for consensus heparin binding motifs identified a seven amino acid epitope on ErbB-3 extracellular ligand binding domain between amino acids lysine 466 and arginine 472. Substituting this region with the corresponding sequence from ErbB-1 yielded a chimeric mutant receptor with no heparin binding ability determined by complete loss of its associated receptor (A), compared to protein A (P.A.) coupled receptors (B). (C) Sequence alignment of the newly identified ErbB3 HSB sequence with known HSB domains of other putative heparin-regulated growth factor receptors (lower panel).

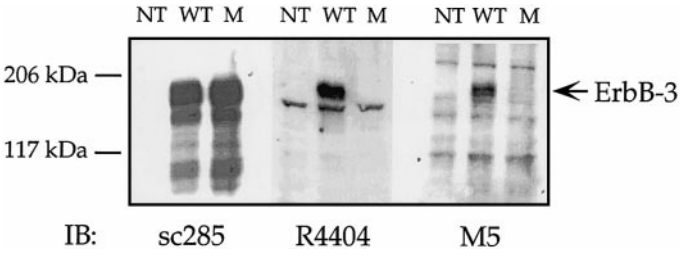

FIG. 4. Specific recognition of ErbB-3 receptors by polyclonal antibodies directed to the identified heparin binding epitope. SDS–PAGE and Western blotting of nontransfected (NT) or wild-type (WT) and heparin binding mutant (M) ErbB-3 receptor expressing cell lysates.

heparin beads at all (Fig. 3B), demonstrating that this specific mutation completely abolished its heparin-binding activity and suggesting that this restricted linear sequence may constitute a major heparin binding epitope on ErbB-3.

To test whether this putative heparin binding epitope of ErbB-3 is surface exposed and available for extracellular interaction we generated site directed antibodies by immunizing both mice and rabbits with a short peptide sharing the putative heparin binding sequence of ErbB-3. The polyclonal antiserum of both mice and rabbits efficiently and specifically bound to the denatured receptor as determined by Western immunoblotting (Fig. 4), as well as to the native, nondenatured soluble receptor as determined by an ELISA assay (not shown). None of these antibody preparations recognized the mutated receptor lacking this heparin binding domain that was, however, indistinguishably recognized by the commercially available anti-ErbB-3 receptor antibodies (Fig. 4). These results strongly suggest that the identified major heparin binding epitope is surface exposed and available for interactions with site directed antibodies.

DISCUSSION

Heparan sulfates (HS), more than all other glycosaminoglycans, have been shown to participate in vital biological processes from early development to adult life of multicellular organisms. More recently, these specifically modified polysaccharides, arranged in sulfated domains were found to interact primarily and with high affinity with a variety of enzymes and regulatory proteins, such as growth factors and cytokines. These highly specific interactions constitute a major mechanism regulating growth factor–receptor interactions *in vitro* and *in vivo,* which once disturbed can readily lead to a loss of function and human pathology.

In general, it is conceivable to divide HS binding (HSB) proteins or protein modules into two major categories based on their primary and secondary structure and their functional consequences. The most common HSB motifs are those serving as retention signals responsible primarily for localizing and concentrating the protein to a restricted region of specific tissues or cells; the second and most recently discovered category includes functional, regulatory elements directly involved in modulating the biological activity of the associated protein.

As retention signals, HSB sites are usually, but not always, composed of long stretches, typically of several clusters each of three basic residues or more in a linear protein sequence. These are most frequently found at either the N- or C-terminal regions of the protein, extending away from a functional, receptor binding core. Retention signals, present on multiple signaling molecules such as IFNγ, VEGF 189, PDGF BB, chemokines, such as SDFIα and others, provide a strong anchor to cell-surface and extracellular matrix resident HS proteoglycans, and serve to concentrate these factors at their site of action.

Regulatory HSB domains, on the other hand, are in most cases non-linear, conformational epitopes where several basic residues from different regions of the protein join together to assemble a defined, highly specific HSB interface. Such motifs are present on multiple heparin-dependent growth factor and cytokines, such as FGFs 1–23, VEGF 165, hepatocyte growth factor, midkine, and others. The proper folding of such protein modules is, therefore, critical for HS-dependent high affinity binding and receptor activation. Both FGF through its compact tri-fold symmetry and VEGF 165 through its defined cysteine rich module, and in contrast to the retention type of HSB proteins, completely lose their heparin affinity upon destruction of their secondary structure.

Most recently, HSB sites have been identified within the extracellular ligand binding domains of growth factor receptors. First identified on FGFR1 (9) and shared by all FGF receptors, these HSB sites constitute an essential element in the receptor high affinity, ligand binding domain and are required for the receptor signal transduction. These relatively short epitopes spanning between 7 to 10 amino acids in length, like the one described here for the ErbB3 receptor tyrosine kinase represent a novel class of regulatory, albeit linear, HSB motifs. In contrast to the linear HSB retention signals which contain at least three basic residues in tandem or more, such long basic clusters are rarely found in the linear receptor type of regulatory HSB motifs. These domains, on the other hand, seem to involve three to four positively charged foci of one or two basic residues each, spaced by one or two most frequently non-charged amino acids (Fig. 3C).

The overall charge density of these regulatory HSB motifs, based on sequence analysis of several such motifs on FGFRs, VEGFRs, ErbB3 and other growth factor receptors (Fig. 3C) and calculated as the ratio of basic to non-charged residues, B/X is close to or equal to 1, which is in marked contrast to that of the typical,

highly charged, retention signals with their long stretches of basic residues where the B/X ratio is always much larger than 1. It is interesting to note that heparin affinity at least of these receptors is probably affected mostly by the charge distribution rather than by charge density as evidenced for example by the lack of any apparent heparin affinity or the soluble form of VEGFR2 (KDR) which shows a significantly higher charge density than VEGFR1 (flt1) which has a B/X ratio of less than 1 in its putative HSB region (Fig. 3C). These restrictions, together with the unique charge density and distribution pattern of sulfate groups on the associated heparan sulfate most likely allow for the specific, but nevertheless dissociable interactions, required for the fine tuning of the highly regulated receptor activities. Such motifs may also be involved in recruiting and clustering of receptors at particular sites on the cell surface, such as focal adhesion contacts, where several HSPGs have been localized.

All FGF receptors contain regulatory HSB elements in a restricted region localized to the N-terminal part of their second IgG-like domain. These fulfill the above criteria being BXXBBXB for FGFRs 1 and 2; BXXBXXBB for FGFR3 and BXXBXXBBXB for FGFR4. Structural analysis of the recently resolved costructures of FGF receptors 1 and 2 with their ligands and a heparin fragment suggest that these linear regulatory HSB motifs constitute one out of three β-strands directly involved in heparin binding. The above suggested motif seems to be the major one as it coordinates with at least two sulfate groups on the polysaccharide one of which is the 6-0-sulfate which has been implicated to be specifically required for biologically relevant HS-FGF-R interactions (31). The ErbB3 receptor tyrosine kinase contains a single linear HSB motif of BBXBXXB and is different from any previously described consensus for HSB sequences and which is not present in any of the other three members of this receptor family. This highly charged motif is available for interaction both with heparin and with monoclonal antibodies recognizing the native protein. The biological role of ErbB3-HS association, however, has yet to be determined.

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REFERENCES

1. Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000) *EMBO J.* **19,** 3159–3167.
2. Sierke, S. L., Cheng, K., Kim, H. H., and Koland, J. G. (1997) *Biochem. J.* **322,** 757–763.
3. Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., 3rd. (1994) *J. Biol. Chem.* **269,** 14661–14665.
4. Alimandi, M., Romano, A., Curia, M. C., Muraro, R., Fedi, P., Aaronson, S. A., Di Fiore, P. P., and Kraus, M. H. (1995) *Oncogene* **10,** 1813–1821.
5. Chen, X., Yeung, T. K., and Wang, Z. (2000) *Biochem. Biophys. Res. Commun.* **277,** 757–763.
6. Tzahar, E., Levkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D., and Yarden, Y. (1994) *J. Biol. Chem.* **269,** 25226–25233.
7. Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. (1991) *Cell* **64,** 841–848.
8. Rapraeger, A. C., Krufka, A., and Olwin, B. B. (1991) *Science* **252,** 1705–1708.
9. Kan, M., Wang, F., Xu, J., Crabb, J. W., Hou, J., and McKeehan, W. L. (1993) *Science* **259,** 1918–1921.
10. Habuchi, H., Suzuki, S., Saito, T., Tamura, T., Harada, T., Yoshida, K., and Kimata, K. (1992) *Biochem. J.* **285,** 805–813.
11. Ornitz, D. M., Yayon, A., Flanagan, J. G., Svahn, C. M., Levi, E., and Leder, P. (1992) *Mol. Cell Biol.* **12,** 240–247.
12. Maccarana, M., Casu, B., and Lindahl, U. (1993) *J. Biol. Chem.* **268,** 23898–23905.
13. Pantoliano, M. W., Horlick, R. A., Springer, B. A., Van Dyk, D. E., Tobery, T., Wetmore, D. R., Lear, J. D., Nahapetian, A. T., Bradley, J. D., and Sisk, W. P. (1994) *Biochemistry* **33,** 10229–10248.
14. Brickman, Y. G., Ford, M. D., Small, D. H., Bartlett, P. F., and Nurcombe, V. (1995) *J. Biol. Chem.* **270,** 24941–24948.
15. Li, L. Y., Safran, M., Aviezer, D., Bohlen, P., Seddon, A. P., and Yayon, A. (1994) *Biochemistry* **33,** 10999–10107.
16. Safran, M., Eisenstein, M., Aviezer, D., and Yayon, A. (2000) *Biochem. J.* **345,** 107–113.
17. Krufka, A., Guimond, S., and Rapraeger, A. C. (1996) *Biochemistry* **35,** 11131–11141.
18. Wong, P., Hampton, B., Szylobryt, E., Gallagher, A. M., Jaye, M., and Burgess, W. H. (1995) *J. Biol. Chem.* **270,** 25805–25811.
19. Dougher-Vermazen, M., Hulmes, J. D., Bohlen, P., and Terman, B. I. (1994) *Biochem. Biophys. Res. Commun.* **205,** 728–738.
20. Roeckl, W., Hecht, D., Sztajer, H., Waltenberger, J., Yayon, A., and Weich, H. A. (1998) *Exp. Cell Res.* **241,** 161–170.
21. Tessler, S., Rockwell, P., Hicklin, D., Cohen, T., Levi, B. Z., Witte, L., Lemischka, I. R., and Neufeld, G. (1994) *J. Biol. Chem.* **269,** 12456–12461.
22. Rahmoune, H., Rudland, P. S., Gallagher, J. T., and Fernig, D. G. (1998) *Biochemistry* **37,** 6003–6008.
23. Aviezer, D., Levy, E., Safran, M., Svahn, C., Buddecke, E., Schmidt, A., David, G., Vlodavsky, I., and Yayon, A. (1994) *J. Biol. Chem.* **269,** 114–121.
24. Cardin, A. D., and Weintraub, H. J. (1989) *Arteriosclerosis* **9,** 21–32.
25. Sobel, M., Soler, D. F., Kermode, J. C., and Harris, R. B. (1992) *J. Biol. Chem.* **267,** 8857–8862.
26. Flanagan, J. G., and Leder, P. (1990) *Cell* **63,** 185–194.
27. Ornitz, D. M., Herr, A. B., Nilsson, M., Westman, J., Svahn, C. M., and Waksman, G. (1995) *Science* **268,** 432–436.
28. Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J., and Rees, D. C. (1996) *Science* **271,** 1116–1120.
29. Margalit, H., Fischer, N., and Ben-Sasson, S. A. (1993) *J. Biol. Chem.* **268,** 19228–19231.
30. Hileman, R. E., Fromm, J. R., Weiler, J. M., and Linhardt, R. J. (1998) *Bioessays* **20,** 156–167.
31. Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U., and Rapraeger, A. C. (1993) *J. Biol. Chem.* **268,** 23906–23914.