Characterization of Sequences within Heparin-binding EGF-like Growth Factor That Mediate Interaction with Heparin*

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Heparin-binding (HB) epidermal growth factor (EGF)-like growth factor (HB-EGF), a member of the EGF protein family, is a potent mitogen for fibroblasts, smooth muscle cells, and keratinocytes that was initially identified as a secreted product of macrophage-like cells. HB-EGF and EGF appear to act on target cells utilizing the same receptor, but HB-EGF is distinguishable from EGF by its strong affinity for heparin. To facilitate studies of structure-function relationships in HB-EGF, a bacterial recombinant expression system was established that produced biologically active HB-EGF with the expected disulfide bonding pattern. Mutagenesis and protease digestion studies of the recombinant HB-EGF, coupled with heparin-binding analyses of synthetic peptides, indicated that the sequences within HB-EGF mediating its interaction with heparin are located primarily in a stretch of 21 amino acids characterized by a high content of lysine and arginine residues. Most of this heparin-binding domain lies in an amino-terminal region of HB-EGF that has no counterpart in EGF, but a portion of the 21-residue sequence extends into the EGF-like region of HB-EGF. In addition, the mutagenesis and synthetic peptide studies indicated that sequences in HB-EGF lying outside of the 21-residue stretch can also influence the interaction with heparin. Finally, a synthetic peptide derived from the 21-residue stretch was found to compete with HB-EGF for binding to Chinese hamster ovary cells, suggesting that the heparin-binding sequences in HB-EGF may also mediate the interaction of this factor with cell surface heparan sulfate proteoglycan.

Several new mitogens in the epidermal growth factor (EGF)1 protein family have recently been identified that display the ability to bind the glycosaminoglycan, heparin (1-7). Among these is the mitogen known as heparin-binding EGF-like growth factor (HB-EGF), which elutes from heparin-Sepharose columns at about 1.0-1.2 M NaCl and which was first identified as a secreted product of cultured human monocytes, macrophages, and the monocyte-like U-937 cell line (4, 8). Analysis of purified, U-937-derived HB-EGF indicated that the secreted factor is heterogeneous in structure but can extend at least 86 amino acids and contain two sites of O-linked glycosylation (4, 9). The carboxyl-terminal half of the secreted HB-EGF shares approximately 35% sequence identity with human EGF, and includes six cysteines spaced in the pattern characteristic of members of the EGF protein family (4). HB-EGF has been shown to interact with the same high affinity receptors as EGF on bovine aortic smooth muscle cells and A431 epidermoid carcinoma cells (4, 9, 10), strongly suggesting that the EGF-like region in HB-EGF is the site of the high affinity receptor-binding domain. In contrast, the amino-terminal portion of the mature factor, which is characterized by stretches of hydrophilic residues, has no structural equivalent in the 53 residues of EGF. Since EGF displays little if any ability to bind heparin (10, 11), the amino-terminal portion of HB-EGF has been suggested as the probable site of the heparin-binding domain within this factor (9).

The ability of heparin-binding growth factors to interact with heparin appears in general to be a reflection of a physiologically more relevant interaction occurring in vivo between these factors and heparan sulfate proteoglycan molecules, which are found on the surface of cells and in extracellular matrix (12). The biological consequences of this in vivo interaction have been best studied for mitogens in the fibroblast growth factor (FGF) protein family, particularly basic FGF (FGF-2) and acidic FGF (FGF-1). A number of recent studies (13-20) have indicated that FGF-2 and FGF-1 cannot bind their receptors or activate a cellular response unless heparin or heparan sulfate molecules are present. These results have led to the proposal that an initial, requisite step for signal transduction by the FGFs may be the formation of a trimolecular complex involving an FGF ligand, a tyrosine kinase receptor molecule, and heparan sulfate proteoglycan (13, 14).

1The abbreviations used are: EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; TE, 20 mM Tris-HCl (pH 7.5), 5 mM EDTA; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; BSA, bovine serum albumin; EGF-D, EGF-like domain; CHO, Chinese hamster ovary; TGF-α, transforming growth factor-α; FPLC, fast protein liquid chromatography.
16, 18-21). In contrast, low affinity binding of the FGFs to heparan sulfate molecules alone can occur in the absence of the high affinity receptors (13, 22, 23).

Binding to heparin-like molecules has also been shown to have significant consequences for growth factors that do not belong to the FGF protein family. For example, incorporation of a highly basic stretch of amino acids into the A chain of platelet-derived growth factor (PDGF) through alternative mRNA splicing appears to create a form of this mitogen that remains localized to cell surface heparan sulfate, rather than being freely secreted and diffusible (24, 25). A similar phenomenon may occur with the 189- and 206-residue alternatively spliced forms of the related mitogen, vascular endothelial growth factor (VEGF) (26). In addition, experiments utilizing heparinase-treated endothelial cells have indicated that VEGF is similar to FGF-1 and FGF-2 in requiring the presence of heparin-like molecules for binding to its high affinity receptors (27). Alternatively, receptor binding by some growth factors can be inhibited by the presence of heparin-like molecules, as has been demonstrated for the EGF-like mitogen known as amphiregulin (2, 28). Overall, the results indicate that the heparin/heparan-binding growth factors bind to sites that are agonist specific. Further experiments indicate that the ultimate biological activity and bioavailability of these factors may depend not only on the spatial and temporal expression of the factors and their respective high affinity receptors, but also on the local expression of the heparan sulfate proteoglycans (13, 14, 29).

Given the importance of heparin-like molecules in modulating the activity of other heparin-binding growth factors, it seemed likely that the actions of HB-EGF would also be modulated by its interaction with these glycosaminoglycans. Such modulation may explain why HB-EGF, despite its apparent use of the same high affinity receptors as EGF on bovine aortic smooth muscle cells (10), has been observed to be a significantly better mitogen for these cells (4). To begin to characterize the HB-EGF-heparin interaction in greater detail, we have made a series of synthetic peptides and have noted that while much of the interaction sequence within the protein, residues lying within the EGF-like domain, play a role in heparin binding.

EXPERIMENTAL PROCEDURES

Recombinant Expression of HB-EGF in Escherichia coli—Recombinant forms of HB-EGF were produced in a bacterial expression system using the plasmid pNA28 (Fig. 1). In this plasmid, the coding region for a 75-amino acid fragment of human HB-EGF is fused in-frame with a sequence encoding a modified version of the first 73 amino acids of the Trp9 chromomelanocytic acid transferase (30). A methionine codon was placed in the vector between the chromomelanocytic acid transferase and the HB-EGF coding sequences to provide a site for cyanogen bromide cleavage within the encoded fusion protein. Expression of the chromomelanocytic acid transferase-HB-EGF fusion driven in pNA28 by a sequence corresponding to the E. coli trp promoter, operator, and ribosome binding site (residues -56 to +22 of the trp operon; see Ref. 31).

E. coli cells of strain W3110 (F- IN(rrnD-rrnE) carrying pNA28 were grown at 37 °C in a supplemented minimal medium (M9 medium (32) containing 0.4% glucose, 2 mg/ml thiamine, 0.8 mM MgSO4, 1% casein hydrolysate, 4 mm L-tryptophan, and 6.25 g/ml tetracycline) to an OD600 of 0.5-0.7. Transcription from the trp promoter was then induced by the addition of 125 μM isopropylthiogalactoside (IPTG) to the cultures to a final concentration of 30 μg/ml of IPTG. After overnight incubation at 37 °C, the cells were harvested by centrifugation, and the cell pellet was resuspended in TE containing 0.5 μM Tris-HCl, pH 7.5, 5 mM MgCl2, and 5 mM EDTA containing 2 μg/ml ampicillin (1 mg/ml chloramphenicol). The cells were then resuspended in TE containing 1 μM, and the phase-bright inclusion bodies that had formed in the cells were collected by centrifugation.

Purification of Recombinant HB-EGF—Inclusion bodies were resuspended in 70% formic acid (8-10 ml/g of wet weight of the inclusion bodies). To cleave the HB-EGF sequence out of the fusion protein, cyanogen bromide (Sigma; 50 mg/g of wet weight of inclusion bodies) was added and the suspension was stirred under argon for 4 h at room temperature. The reaction mixture was dried under vacuum, resuspended in TE containing 6 μM urea, and then loaded onto an SP-Sephadex C25 column (Pharmacia LKB Biotechnology Inc.) equilibrated with TE containing 6 μM urea and 0.1 mM NaCl. Bound proteins were eluted with TE containing 6 μM urea and 0.6 mM NaCl. To allow the recombinant HB-EGF to refold and form its disulfide bonds prior to final purification, the eluate from the SP-Sephadex column was supplemented with glutathione and glutathione disulfide (Boehringer Mannheim) to concentrations of 6 and 12 mM, respectively, and the resulting solution was diluted with 5 volumes of 20 mM Tris-HCl, pH 8.8, 5 mM EDTA. The refolding mixture was allowed to stand at 4 °C for at least 4 h and was then loaded onto a heparin-Sepharose column (Pharmacia) equilibrated with TE containing 0.1 mM NaCl. Bound proteins were step-eluted first with TE containing 0.6 mM NaCl, and then with TE containing 2 mM NaCl. Final purification of the recombinant HB-EGF was achieved by loading the 2 mM NaCl eluate onto a semi-preparative (1 cm × 25 cm) Void C18, or C18 reversed-phase high performance liquid chromatography
(HPLC) column equilibrated with 15% acetonitrile in 0.1% trifluoroacetic acid, and then eluting bound protein at a flow rate of 4 ml/min with a 40-min gradient of 15-35% acetonitrile in 0.1% trifluoroacetic acid. Protein elution from the column was monitored by absorbance at either 214 or 220 nm. The major absorbance peak eluting at approximately 25% acetonitrile was collected and shown by amino-terminal amino acid sequence analysis to represent the wild-type HB-EGF.

Thermolysin Fragment Analysis—Isolation of thermolysin peptide fragments was carried out using methods similar to those previously described (33, 34). An aliquot (50-200 μg) of recombinant HB-EGF was dried under vacuum, resuspended in phosphate-buffered saline (PBS) (20 mM Tris-HCl, pH 7.5) at a concentration of 1.33 mg/ml, and then mixed with 2.4 volumes of 0.1 M pyridine acetate (pH 6.5). Thermolysin (Boehringer Mannheim) was added to the mixture such that the final ratio (w/w) of enzyme to recombinant protein was 1:50. After incubation at 37 °C for 48 h, the reaction mixture was loaded at a flow rate of 1 ml/min onto a Vydac C4 HPLC column (0.46 cm × 25 cm) equilibrated with 0.1% trifluoroacetic acid. The column was washed for 5 min with 0.1% trifluoroacetic acid, and peptide fragments were then eluted with a 60-min gradient of 0% to 40% acetonitrile in 0.1% trifluoroacetic acid. Peptide elution from the column was monitored by absorbance at 214 nm. Absorbance peaks detected in the eluate were collected for amino acid sequence analysis.

Amino-terminal Amino Acid Sequence Analysis—Amino-terminal sequences of recombinant HB-EGF samples and thermolysin digestion products were established using an Applied Biosystems 477A gas-phase protein sequenator with an on-line Applied Biosystems model 120A phenylthiohydantoin amino acid analyzer. Purification of U-937-derived HB-EGF—HB-EGF was isolated as described previously (4, 9) from medium conditioned by U-937 cells. The protein concentration of the purified material (combination of "peak 1" and "peak 2" forms of HB-EGF; see Ref. 9) was determined by amino acid analysis.

Mitogenesis Assay—Relative mitogenic activity of recombinant and U-937 derived HB-EGF samples was judged using the mouse fibroblast-like cell line, AKR-2B (35). The AKR-2B cells were plated in 96-well plates at a density of 2000 cells/well in 0.2 ml/well of McCoy's 5A medium (Life Technologies, Inc.) supplemented with 5% fetal bovine serum (HyClone) and 10 μg/ml gentamicin sulfate (Sigma). The plated cells were then incubated at 37 °C for 7 days. Serial dilutions of growth factor preparations to be tested were made with a diluent consisting of serum-free McCoy's 5A medium supplemented with 5 μg/ml bovine insulin (Sigma), 50 μg/ml bovine serum albumin (BSA; Sigma), and 10 μg/ml gentamicin sulfate. Aliquots (50 μl/well) of the serial dilutions were added directly to the cells in the 96-well plates without a change of medium. Control wells received 50 μl of the diluent solution with no added HB-EGF. After incubation of the plates for 24 h, each well received 1 μCi of [methyl-3H]thymidine (Du Pont) and the thymidine incorporation was then continued for an additional 3 h. The medium in each well was removed, and the cells were fixed for 10 min on ice with 0.3 ml/well of cold 10% trichloroacetic acid. After two washes with cold 10% trichloroacetic acid, the cells were solubilized with 0.15 ml/well of 0.2 M NaOH containing 40 μg/ml herring sperm DNA. A 0.1 ml aliquot from each well was then removed for scintillation counting.

Isolation of EGF-D (32-75)—Recombinant HB-EGF was lyophilized to dryness, resuspended in PBS to a concentration of 1-2 mg/ml, and digested with 1-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin ( Worthington) at a final HB-EGF-to-protease ratio (w/w) of 100:1. After 1 h at room temperature, the digestion mixture was loaded onto a prepacked Vydac C4 HPLC column and eluted with a 40-min gradient of 15% to 35% acetonitrile in 0.1% trifluoroacetic acid. EGF-D (32-75), a digestion product containing residues 32-75 (EGF-D (32-75)), was collected and shown by amino acid analysis to represent the wild-type HB-EGF.

Peptide Synthesis—Synthetic peptides were prepared using solid phase synthesis procedures (38) on an Applied Biosystems 430A automated peptide synthesizer. To generate a single disulfide bond in a peptide, the synthesis of the peptide was carried out using cysteine residues with a 4-methylbenzyl protecting group on the side chain; the disulfide bond was formed by first deblocking the peptide with a hydrogen fluoride solution and then oxidizing the peptide at a concentration of less than 1 mg/ml with 10 mM potassium ferricyanide. For peptide HB-EGF (32-60)-NH2 containing two disulfide bonds, the cysteines corresponding to residue positions 36 and 49 were initially incorporated into the synthetic peptide with 4-methylbenzyl protecting groups, while the cysteines corresponding to positions 44 and 60 were incorporated with acetalomidomethyl protecting groups. The disulfide bond between cysteines 36 and 49 was formed as described above with potassium ferricyanide oxidation after hydrogen fluoride treatment to remove the 4-methylbenzyl protecting groups. Following gel filtration of the peptide on a G25F column, the disulfide bond between cysteines 44 and 60 was formed using iodine deblocking and oxidation as described elsewhere (39). Synthetic peptides were purified by a combination of cation exchange chromatography on CM-Sepharose (Pharmacia) and reversed-phase HPLC on a preparative Vydac C4 column. The purified peptides were characterized by amino acid analysis and in some cases by amino acid sequencing. The disulfide binding pattern in HB-EGF (32-60)-NH2 was confirmed by sequencing of thermolysin digestion products, as described above.

Peptide Competition of HB-EGF Binding to Chinese Hamster Ovary (CHO) Cells—CHO-K1 cells were grown to confluence in 6-well plates in Ham's F-12 medium (Life Technologies, Inc.) supplemented with 10% bovine calf serum (Life Technologies, Inc.) and 2 μg/ml-glutamine (Sigma). The cells were washed three times with ice-cold binding buffer (Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 20 mM HEPES, pH 7.4 and 0.2% BSA), and were then chilled on ice for 10 min in 750 μl/well of binding buffer. Serial dilutions of the synthetic peptide HB-EGF (21-33) were added in 7.5-μl aliquots to duplicate wells. [3H]-HB-EGF (7.5 μl), prepared as described previously (9), was then added to each well, and the plates were incubated for 2 h at 4 °C. After two washes with binding buffer and three washes with PBS, the cells were exposed three times to PBS containing 0.2% BSA and 2 mM NaCl. The three high salt washes of each well were collected and pooled, and the amount of [3H]-present in each pool was determined with a γ counter.

RESULTS

Isolation and Characterization of Bacterial Recombinant HB-EGF—To allow for the use of site-directed mutagenesis techniques in defining the heparin-binding sequences in HB-EGF, a recombinant expression system was developed for the factor (see Fig. 1 and "Experimental Procedures"). A 75-amino acid form of human HB-EGF (HB-EGF31, corresponding to residues 73 through 147 of the 208-residue HB-EGF primary translation product) was chosen for expression because it represented the sequence initially determined for the major form of HB-EGF detected in U-937 conditioned medium (9). When the recombinant HB-EGF31 was obtained from the final HPLC step of the purification scheme (see "Experimental Procedures") was subjected to amino acid sequencing, a single NH2-terminal peptide sequence (RVTLSSKQPA ... ) was detected. A molecular weight of 8484.4 was predicted if the purified product retained all 75 residues and had formed three disulfide bonds; electrospray ionization mass spectrometry of the purified material (performed by M-Scan Inc., West Chester, PA) indicated a molecular weight of 8484.8 ± 0.9, in good agreement with the predicted value.

Analysis of the disulfide bond arrangement in mouse submaxillary gland EGF (33) and in recombinant human trans-
forming growth factor-α (TGF-α) (34) had demonstrated in both of these related proteins that the first cysteine in each protein is linked to the third cysteine, the second cysteine is linked to the fourth, and the fifth cysteine is linked to the sixth. To determine whether a similar pattern of disulfide bonds was present in HB-EGF₇₅, the protein was digested with thermolysin, and the resulting peptide fragments were separated by reversed-phase HPLC (Fig. 2A). When the thermolysin digestion reaction was treated with 10 mM dithiothreitol to reduce disulfide bonds prior to chromatography on the HPLC column, the peaks labeled 1, 2, and 3 in Fig. 2A were no longer detected in the chromatogram, and multiple smaller peaks appeared (data not shown). Peaks 1, 2, and 3 were therefore collected and subjected to amino acid sequence analysis. As shown in Fig. 2A, the sequencing revealed the presence of two peptides in each of the peaks. Although cysteine residues themselves could not be detected with the sequencing methods employed, a comparison of the sequence of HB-EGF₇₅ (Fig. 1) with the sequence data obtained from the thermolysin digestion products indicated that the two peptides in peak 1 contained the cysteines at residue positions 62 and 71; the two peptides in peak 2 contained cysteines 36 and 49; and the two peptides in peak 3 contained cysteines 44 and 60. These results thus indicate that the disulfide bonding pattern in the EGF-like domain of HB-EGF₇₅ matches that reported for EGF and TGF-α (33, 34).

Since recombinant proteins produced as described above in E. coli will not be glycosylated by the host cell, analyses were carried out to determine whether the lack of O-linked glycosylations on the recombinant HB-EGF₇₅ affected either the strength of binding to heparin or the biological activity of the factor. Using a TSK-heparin 5PW column on a fast protein liquid chromatography (FPLC) system, U-937-derived HB-EGF elutes at between 1.0 and 1.2 M NaCl (9); under the same conditions, HB-EGF₇₅ was found to elute at approximately 1.2 M NaCl (data not shown). In an in vitro assay using AKR-2B cells (Fig. 2B), the recombinant protein stimulated [³H]thymidine incorporation with a similar specific activity and to the same extent as U-937-derived HB-EGF. Similar results were obtained when the recombinant and U-937-derived HB-EGF preparations were assayed in a BALB/c ST3 assay described previously (4, 9) (data not shown).

Characterization of a Proteolytic Fragment of HB-EGF₇₅

Spanning the EGF-like Domain—Initial comparisons between the sequences of EGF and U-937-derived HB-EGF had suggested that HB-EGF might consist of two separate functional domains: a carboxyl-terminal domain (residues 36-71 in Fig. 1) displaying sequence and structural homology to EGF, and therefore most likely containing the site of interaction with the EGF receptor; and an amino-terminal, hydrophilic domain (residues 1-35) not shared with EGF and proposed as the probable site of the heparin-binding domain of the protein (4, 9). In particular, since the analysis of other heparin-binding proteins has indicated that heparin-interaction domains often contain high local concentrations of basic amino acids (40, 41), we initially hypothesized that residues 21-27 of the amino-terminal region of HB-EGF (sequence KRKKGK) would play a critical role in HB-EGF’s interaction with heparin.

To probe the domain structure of HB-EGF₇₅, and to determine whether residues 21-27 are indeed central to the heparin-binding ability of the protein, HB-EGF₇₅ was subjected to partial tryptic digestion as described under “Experimental Procedures.” Fractionation of the digestion products on a reversed-phase HPLC column revealed a single major cleavage product along with multiple minor products. The absorbance peak in the column eluate representing the major product was collected and analyzed by SDS-polyacrylamide gel electrophoresis and TSK-heparin HPLC fractionation; the results of these analyses indicated that the absorbance peak corresponded to a homogeneous peptide species (data not shown). NH₂-terminal amino acid sequencing of this peptide yielded a single sequence reading KRDPXL . . . (see Fig. 1). Analysis of thermolysin fragments derived from this peptide confirmed the presence of at least residues 32-71 spanning the EGF-like domain of HB-EGF; since there are no trypsin cleavage sites lying between residues 71 and 75, the peptide was assumed to extend through the serine at the carboxyl terminus of HB-EGF and was accordingly named EGF-D-(32-75). The tryptic digestion results thus indicated that only the EGF-like portion of HB-EGF₇₅ is folded in such a way as to exclude access of trypsin to potential digestion sites.

Fig. 3A shows the elution pattern obtained from a TSK-heparin HPLC column loaded with a mixture of HB-EGF₇₅ and EGF-D-(32-75). Unlike the analysis described above using a TSK-heparin column on an FPLC system, where HB-EGF₇₅ was found to elute with approximately 1.2 M NaCl, an NaCl concentration of about 0.8-0.9 M NaCl was sufficient to elute HB-EGF₇₅ in the HPLC system. In the experiment shown in Fig. 3A, HB-EGF₇₅ eluted at an NaCl concentration of 0.88 M. Surprisingly, even though EGF-D-(32-75) had lost the majority of the amino-terminal region of the protein, it still displayed strong binding to heparin and eluted at 0.66 M NaCl. Thus, about 75% of the heparin binding ability of HB-EGF₇₅
by performing chromatographic analyses under identical conditions of the proteins encoded by the mutated plasmids were almost as high an NaCl concentration as full-length HB-EGF75, suggesting that residues 20 through 42 indeed represent the major heparin binding domain of HB-EGF75.

The mutants that were made are shown schematically in Fig. 4. In each case, between one and three basic amino acids were changed to alanine residues. Chromatographic analyses of the various mutants by TSK-heparin HPLC revealed that mutations in any one of the three stretches of basic residues lowered the NaCl concentration needed to elute the protein from heparin. In some cases, the elution positions of the mutant proteins relative to wild-type recombinant HB-EGF75 were confirmed by co-injection of the mutant and wild-type proteins onto the TSK-heparin column (Fig. 5A). Among the mutants made, the greatest changes in elution position were seen (i) when triple mutations were made in the 31KRKKKGK sequence (K21A/R22A/K23A and K23A/K24A/K25A mutants, eluting at 79–83% the NaCl concentration needed to elute wild-type HB-EGF75); (ii) when a double mutation was made in the 38RKYK sequence (K39A/K41A mutant, eluting at 80% the NaCl concentration needed to elute wild-type); and (iii) when a double mutation was made in the 21KR sequence (K32A/R33A mutant, eluting at 73% the NaCl concentration needed to elute wild-type) (Fig. 4). All of the mutant proteins, including the most severely affected (K32A/R33A), showed essentially wild-type activity in the AKR-2B mitogenic assay (Fig. 5B and data not shown).

Since all of the changes made in the three stretches of basic amino acids resulted in a net decrease in the number of positive charges in HB-EGF, the possibility existed that the heparin interaction was influenced in the mutants by overall charge rather than specific site changes, and that a change in any basic amino acid might be sufficient to result in a drop in the strength of the interaction. To test this possibility, the mutant K16A was made in which a lysine residue lying outside the three stretches of basic amino acids was changed to an alanine (Fig. 4). K16A was found to elute from the TSK-heparin column at essentially the same NaCl concentration as wild-type HB-EGF75, indicating that more than a nonspecific loss of positive charge is needed to disrupt the strength of the heparin interaction with this protein.

A second mutant, K53A, was also constructed with the intent of determining how specific the alterations in heparin binding were to mutations in the 31KRKKKGK, 21KR, and 38RKYK sequences. Unexpectedly, the K53A mutation was found to have as much effect on the strength of the heparin interaction as some of the single-residue changes in the 31KRKKKGK sequence (Fig. 4). The loss in heparin binding strength in the K53A mutant did not appear to be a side effect of general disruption of the folding of the protein, since the mutation did not cause a change in the ability of the protein to stimulate DNA synthesis in AKR-2B cells (Fig. 5B). Taken together, then, the results shown in Fig. 4 indicated that while 21KRKKKGK, 31KR, and 38RKYK are indeed important for the interaction of HB-EGF with heparin, the strength of the heparin interaction can also be influenced by additional sequences in the protein.

Analysis of Synthetic Peptides—If the three stretches of basic amino acids boxed in Fig. 1. provide the bulk of the actual contact points for the interaction of HB-EGF with heparin, then subfragments of HB-EGF containing these stretches would also be expected to bind strongly to heparin. To test this prediction, a series of synthetic peptides were generated representing various portions of the HB-EGF protein (Fig. 6). TSK-heparin chromatography of the peptide [Ser]38HB-EGF-20–42 spanning all three stretches of basic residues revealed that this peptide eluted from the heparin at almost as high an NaCl concentration as full-length HB-EGF75, suggesting that residues 20 through 42 indeed represent the majority of the heparin binding domain of HB-EGF.
Heparin-binding Domain of HB-EGF

Peptides missing part of this region show decreased heparin binding strength: HB-EGF-(21-33) elutes from the TSK-heparin column at between 0.83-0.84 M NaCl. The NaCl concentration needed to elute each mutant from the column was then divided by the NaCl concentration needed to elute wild-type HB-EGF, while peptide [SerM]HB-EGF-(30-42) elutes at 43% the NaCl concentration needed to elute a wild-type HB-EGF sample (chromatographed on the same day), and the resulting quotient was multiplied by 100. The heparin elution result for each mutant is thus expressed as the percent of the NaCl elution concentration for wild-type HB-EGF (Rel. Heparin Elution Conc.). In these experiments, wild-type HB-EGF eluted from the TSK-heparin column at between 0.83-0.84 M NaCl.

DISCUSSION

Given the results of the in vitro mutagenesis and synthetic peptide studies presented here, we propose that the interac-
tion of the 75-amino acid form of HB-EGF with heparin is primarily mediated by a stretch of amino acids extending from residues 21-41 of the protein (Fig. 8A). Although the majority of this 21-residue heparin-binding sequence is located as predicted (9) in the hydrophilic portion of the factor lying amino-terminal to the first cysteine, one of the three stretches of basic amino acids in this sequence (38RKYK) actually lies within the region of HB-EGF sharing homology with EGF. The finding that heparin-binding sequences fall within the EGF-like domain of HB-EGF was unexpected, since EGF itself does not appear to bind heparin at all, even in low salt conditions (11). However, it is important to note that the 38RKYK sequence is not conserved at the corresponding location in human EGF, where the sequence LSHD occurs instead (Fig. 8A). The 38RKYK sequence is also not conserved at the corresponding position in TGF-α, consistent with the observation by Benner et al. (8) that TGF-α like EGF did not bind to a heparin column loaded in low salt.

The in vitro mutagenesis study (Fig. 4) focused on lysine and arginine residues in HB-EGF, since studies on other heparin-binding proteins had demonstrated the general importance of these amino acids in interactions with heparin (40, 41). No attempts were made to mutagenize the non-basic
residues in the proposed 21-residue heparin-binding stretch, and the contribution made by these non-basic residues to heparin binding is thus unclear. Comparison of the human, rat, and mouse forms of HB-EGF (42, 43) revealed that, except for exchanges of lysine for arginine or vice versa, the 21-residue stretch is entirely conserved between these species. Given that the overall sequence conservation (excluding lysine-arginine exchanges) is about 80% between the human and rodent HB-EGFs (42), the complete conservation of the non-basic residues in the 21-residue stretch suggests that they are playing an important role in the biological activity of the growth factor, whether or not they participate in the heparin interaction.

Meari et al. (44) recently reported the construction of chimeric proteins consisting of residues 2 through 46 of HB-EGF linked to the amino-terminal end of fusions of TGF-α and Pseudomonas toxin. Unlike the TGF-α-Pseudomonas toxin parent molecules, which eluted from heparin at about 0.15 M NaCl, the chimeras containing the amino terminus of HB-EGF eluted from heparin at about 0.8 M NaCl. Consistent with the synthetic peptide binding studies described here (Fig. 6), these results indicated that sequences in the amino-terminal half of HB-EGF are sufficient to confer the ability to bind to heparin with nearly as high an affinity as wild-type HB-EGF. Besnert et al. (45) have also reported that synthetic peptides derived from the amino-terminal half of HB-EGF can bind strongly to heparin; specifically, the peptide 38RKRKKG was reported to elute from a TSK-heparin FPLC column at 1.5 M NaCl (150% the NaCl concentration needed to elute U-937-derived HB-EGF), while the peptide 37LRKYK eluted at the same NaCl concentration as U-937 HB-EGF (1 M NaCl). The relatively high concentrations of NaCl needed to elute these short peptides, when contrasted with the NaCl concentrations needed to elute the peptides shown in Fig. 6, indicate that the ultimate contributions made by these short basic residue stretches to the HB-EGF-heparin interaction are strongly influenced by neighboring residues and/or by the three-dimensional structure assumed by these sequences within the protein.

Based on the results presented here, Higashiyama et al. (10) synthesized a 21-residue peptide representing the proposed HB-EGF heparin interaction region, for use as a competitive inhibitor of HB-EGF binding to heparan sulfate proteoglycan. The peptide ("P21") was found to inhibit by about 60% the ability of HB-EGF to stimulate migration in bovine aortic smooth muscle cells; by comparison, heparitinase treatment of the cells caused an 80% reduction in the migration response triggered by HB-EGF (PDGF-stimulated migration, however, was unaffected by the peptide or by heparitinase treatment). P21 was also found to be nearly as effective as heparitinase treatment in blocking the cross-linking of 125I-HB-EGF to its high affinity receptor on the smooth muscle cells, while neither P21 nor heparitinase affected 125I-EGF cross-linking. Given the similarity in the effects of P21 and heparitinase, treatment in blocking the cross-linking of 125I-HB-EGF to its high affinity receptor on the smooth muscle cells used by Higashiyama et al. (10). The latter explanation is supported by preliminary results indicating that the K21A/R22A/K23A and K23A/K24A/K25A mutants and EGF-D(32-75) display specific activities about 2-3-fold lower than HB-EGF, for the stimulation of DNA synthesis in the bovine aortic smooth muscle cells.

Another member of the EGF family that binds both to heparin and to the EGF receptor is amphiregulin (2, 3). As with HB-EGF, amphiregulin is rich in lysine and arginine residues in its amino-terminal half, and shares about 40% sequence similarity with EGF in its carboxyl-terminal half. In the sequence alignment shown in Fig. 8A, only portions of the proposed heparin binding region of HB-EGF appear to be conserved in amphiregulin (note in particular the lack of a sequence similar to 38RKYK between the first and second cysteines of amphiregulin). An alternative sequence alignment, however, where the cysteine matches are ignored (Fig. 8B) suggests a much more striking conservation of the heparin interaction region.

Through a comparison of the heparin-binding regions in apolipoprotein B-100, apolipoprotein E, vitronectin, and platelet factor 4, Cardin and Weintraub (40) identified two sequence motifs that they proposed as consensus sequences for heparin recognition. These sequence motifs are X-B-B-X-B-B and X-B-B-X-X-B-X, where a basic amino acid residue is strongly favored at the "B" positions, and a non-basic residue is usually found at the "X" positions. As shown in Fig. 8C, both of these motifs are present in the proposed heparin-binding region in HB-EGF. It has also been proposed that in some heparin-binding proteins, a "complete" heparin interaction domain is formed by residues lying distant from each other in the primary sequence of the protein that are brought into close proximity by the folding of the protein backbone (40, 41). Such a phenomenon may be occurring in HB-EGF with the lysine at position 53, since mutation of this residue caused a drop in heparin binding strength (Fig. 4).

Structural studies are currently underway to determine the actual location of residue 53 relative to residues 21 through 41 in the three-dimensional configuration of HB-EGF.

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