J. Biochem. 122, 474-480 (1997)

CD9 Antigen Interacts with Heparin-Binding EGF-Like Growth Factor through Its Heparin-Binding Domain[1]

Takahiko Sakuma,*,† Shigeki Higashiyama,* Shigeto Hosoe,† Seiji Hayashi,† and Naoyuki Taniguchi*,[2]

*Department of Biochemistry and †Department of Internal Medicine III, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565

Received for publication, May 2, 1997

Heparin/heparan-sulfate proteoglycan (HSPG) binds to heparin-binding epidermal growth factor-like growth factor (HB-EGF) through its heparin-binding domain (HBD), which consists of 21 amino acid residues (P21). The CD9 antigen also interacts with a membrane-anchored form of HB-EGF (proHB-EGF) and enhances its juxtacrine activity. The CD9 antigen potentiates the juxtacrine activity of both proHB-EGF and proamphiregulin, but has no effect on proTGF-α. While both HB-EGF and amphiregulin contain an HBD, TGF-α does not. This suggests that the HBD of HB-EGF is also involved in CD9 antigen binding. Mutant CHO cells which lack HSPG recovered their capacity to bind to immobilized P21 when transfected with CD9 antigen cDNA. This binding was competitively inhibited by heparin in a dose-dependent manner. The interactions between synthetic peptides corresponding to the extracellular domain of CD9 antigen and the immobilized P21 were analyzed with surface plasmon resonance. The ^{119}VIKEVQEFYKDTYNKLKTKD^{138} sequence of the CD9 antigen is thought to represent the binding site for HB-EGF. The k_D values for heparin/P21 and ^{119}V-D^{138}/P21 were $(2.82\pm0.10)\times10^{-8}$ M and $(3.71\pm0.71)\times10^{-6}$ M, respectively. These results suggest that the ^{119}V-D^{138} sequence of the CD9 antigen is the site which interacts with the HBD and may play an essential role in the upregulation of the juxtacrine activity of proHB-EGF.

Key words: CD9 antigen, Chinese hamster ovary cells, heparan-sulfate proteoglycan, heparin-binding EGF-like growth factor (HB-EGF), surface plasmon resonance.

Heparin-binding epidermal growth factor-like growth factor (HB-EGF) belongs to the EGF family of proteins. HB-EGF shows a strong affinity to heparin and binds to the same receptor as EGF and TGF-α (*1*). While HB-EGF binds to the EGF receptor (EGFR), it also binds to cell surface heparan-sulfate proteoglycan (HSPG). The interaction of HB-EGF with cell surface HSPG is essential for its optimal binding to EGFR and for promoting its growth/migratory activity toward vascular smooth muscle cells (*2*). Mutagenesis and protease digestion of recombinant HB-EGF, coupled with analyses using synthetic peptides and heparin, revealed that a 21-amino acid sequence in the amino-terminal region of the soluble HB-EGF (designated as P21) is responsible for its binding to heparin. In addition, this heparin-binding domain (HBD) also interacts with cell surface HSPG (*3*).

As a member of the EGF family, HB-EGF is synthesized as a membrane-bound precursor protein (proHB-EGF) and is processed to yield a mature soluble form through proteolysis. Interestingly, proHB-EGF is also the diphtheria toxin receptor (DTR) (*4*). The cytotoxicity of diphtheria toxin (DT) is augmented when DTR forms a complex with CD9 antigen. A close association between proHB-EGF and CD9 antigen was confirmed by immunoprecipitation and chemical crosslinking (*5*). Furthermore, when both human proHB-EGF and simian CD9 cDNAs were co-expressed in mouse L cells, the juxtacrine growth factor activity of proHB-EGF was significantly up-regulated by approximately 30-fold over that of L cells, which express only proHB-EGF (*6*). Therefore, it can be concluded that CD9 antigen represents an additional modulating factor of proHB-EGF.

While the site of binding between HB-EGF and heparin was determined in detail, the site(s) of association between proHB-EGF and CD9 antigen remains unknown. Elucidation of the proHB-EGF/CD9 antigen interaction site may aid in our understanding of the roles of CD9 antigen in the signal transduction of proHB-EGF, as well as its regulation.

HB-EGF, like TGF-α and amphiregulin, is an autocrine growth factor for human keratinocytes (*7-9*). The HB-EGF-stimulated growth of keratinocytes was inhibited by a monoclonal antibody against CD9 antigen (*10*). Moreover,

[1] This work was supported, in part, by Grants-in-Aid for Cancer Research and Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Tel: +81-6-879-3421, Fax: +81-6-879-3429, E-mail: Seika@biochem.med.osaka-u.ac.jp

Abbreviations: FGF, fibroblast growth factor; CHO cells, Chinese hamster ovary cells; DPBS(−), Dulbecco's phosphate-buffered saline without Mg^{2+}/Ca^{2+}; DT, diphtheria toxin; DTR, diphtheria toxin receptor; EGF, epidermal growth factor; EGFR, EGF receptor; HBD, heparin-binding domain; HB-EGF, heparin-binding EGF-like growth factor; HSPG, heparan-sulfate proteoglycan; SPR, surface plasmon resonance; TGF, transforming growth factor.

when CD9 antigen was transiently expressed in mouse L cells, the juxtacrine activity of proHB-EGF and proamphiregulin was augmented, but that of proTGF-α was not (*10*). This prompted us to hypothesize that the HBD of HB-EGF and the extracellular domain of CD9 antigen may represent the mutual binding site, since HB-EGF and amphiregulin have an HBD, while TGF-α does not. The P21-peptide/CD9 antigen interaction was analyzed by means of surface plasmon resonance (SPR), using synthetic peptides corresponding to the HBD of HB-EGF and the extracellular domain of CD9 antigen. One peptide (^{119}VIKEVQEFYKDTYNKLKTKD^{138}) exhibited specific binding with the P21-peptide and thus represents a possible site of interaction with the HBD of HB-EGF.

MATERIALS AND METHODS

Cells—CHO K1 cells were obtained from the American Type Culture Collection (CCL-61). CHO *pgsD*-677 cells, mutant cells lacking HSPG (*11*), were generously supplied by Dr. J.F. Esko (Department of Biochemistry, University of Alabama, Birmingham, AL). Both cell types were maintained in Ham's F12 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and penicillin G (50,000 U/liter)/streptomycin sulfate (100 mg/liter) under a humidified 5% CO_2 atmosphere.

Adhesion of CHO Cells to P21-Coated Plates—A synthetic 21-amino acid peptide (P21: KRKKKGKGLGKKRDPCLRKYK), which corresponds to the HBD of human HB-EGF (*3*), was synthesized using fluorenylmethoxycarbonyl (Fmoc)-chemistry and purified by HPLC to 95% purity (Biosynthesis, Lewisville, TX). Twelve-well multiwell plates were coated with the P21-peptide as follows: 1 ml of the P21-peptide (5 μg/ml) in a 0.05 M sodium-carbonate/bicarbonate buffer (pH 9.6) was added to each well and the plates were then incubated overnight at 4°C. Unbound P21-peptide was washed out by rinsing twice with 1 ml of Dulbecco's phosphate-buffered saline without Mg^{2+}/Ca^{2+} [DPBS(−)]. The cells to be tested were harvested with 0.5 mM EDTA in DPBS(−) and gentle pipetting to prevent inadvertent damage to cell-surface proteins, and were then added to the P21-coated plates [10^5 cells/well in 1 ml DPBS(−)]. The plates were continuously rocked on a horizontal rotary shaker (~250 cycle/min) at room temperature to avoid non-specific binding of the cells to the plates. The attached cells were harvested by trypsinization and counted with a Coulter counter (Industrial D Model Coulter Electronics, Luton, Beds, UK).

CD9 antigen was transiently expressed in CHO *pgsD*-677 cells by electroporation. Green monkey CD9 antigen cDNA (*12*), inserted into the pRC/CMV vector (Invitrogen, San Diego, CA), was a gift from Dr. E. Mekada (Kurume Univ.). One million CHO *pgsD*-677 cells and an expression plasmid (50 μg) were suspended in HeBS (20 mM Hepes pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose) in an 800 μl cuvette and then subjected to electroporation with a GenePulser (BioRad Laboratories, Hercules, CA) at 250 V and 960 μFD. The cells were then cultured in F12 medium for several hours and dead cells were removed by changing the medium. After 48 h of incubation, the transfected cells were harvested with EDTA/DPBS(−) and used for the experiments, described below.

Inhibition of Adhesion of CD9-Expressing Cells to the Immobilized P21-Peptide by Heparin—The adhesion of CD9-transfected CHO cells to P21-coated plates was measured in the presence of various concentrations of heparin (average MW 19,000 Da; from porcine intestine mucosa, Fuso Pharmaceutical, Osaka) to determine whether heparin is capable of interfering with the binding between CD9 antigen and the HBD of the HB-EGF. CD9 antigen cDNA was transiently expressed in CHO *pgsD*-677 cells and the binding of cells to the P21-coated plates was then assayed as above.

Analysis of P21/Heparin Binding with SPR—The interaction of heparin with the P21-peptide was assayed by means of SPR (*13, 14*) using a BIACORE 2000 Instrument (Biosensor, Uppsala, Sweden). The P21-peptide was immobilized according to the manufacturer's recommended protocol. Conventional amine coupling was avoided, since multiple lysine residues, which may be involved in the association with heparin, would be modified by the immobilization chemistry. The P21-peptide was coupled *via* thiol groups to a CM5 Sensor Chip that had been treated with *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide (EDC) and 2-(2-pyridinyldithio)ethaneamine (PDEA) thiol-coupling reagent (Biosensor). Activation time with NHS/EDC was varied to achieve different levels of immobilization of the P21-peptide. The remaining unreacted reactive groups were blocked with L-cysteine. As a negative control flow cell, we used a sensor chip that was activated/blocked (NHS/EDC/PDEA/cysteine) without immobilizing the P21-peptide. Heparin was diluted in an HBS buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20 [v/v]) and injected at a flow rate of 20 μl/min. The sensor chip surface was regenerated with 1.28 M NaCl. K_D values were evaluated with BIAevaluation software (ver. 2.1, Biosensor). To eliminate the "bulk effect" of the heparin solution, K_D values were determined from steady state binding levels using a multispot sensing (gradient surface) method (*15*). k_d values were also measured with BIAevaluation software, and k_a values were calculated from K_D and k_d values.

Analyses of the P21-/CD9 Antigen Peptide Interaction with SPR—Although CD9 antigen is predicted to have two extracellular domains, the smaller one is short (18–20 amino acids, depending on the species) and is *N*-glycosylated (*16, 17*). Its interaction with HB-EGF would be unlikely. Therefore, eight peptides corresponding to the larger extracellular domain of simian CD9 antigen (*5*) were synthesized in the same manner as for the P21-peptide. These peptides were designed to be 20-amino acids in length with all the peptides overlapping by 10-amino acids (Table I). As a negative control, we employed a sensor chip that was activated/blocked, but with the P21-peptide omitted.

Each peptide was dissolved in HBS, passed through a 0.22 μm-filter and then injected onto P21-immobilized/negative control flow cells at 20 μl/min. The flow cells were regenerated with 0.1 N HCl containing 1 mg/ml L-lysine. The binding between the P21-peptide/CD9-peptides were analyzed with a BIACORE 2000 instrument/BIAevaluation software as for the P21-peptide/heparin analysis.

Sequential Injection of Heparin/CD9 ^{119}VIKEVQEFYKDTYNKLKTKD^{138} Peptide onto the Immobilized P21-Peptide—Since the ^{119}V-D^{138} peptide bound to the P21-peptide

(Table I and Fig. 4C), it was sequentially injected after heparin (0.5 μg/ml) onto a P21-immobilized sensor chip at various concentrations (0 μg/ml [running buffer], 250 μg/ml, and 500 μg/ml) to verify that the ^{119}V-D^{138} peptide and heparin commonly bind to the P21-peptide. In order to confirm the results, the sequence of injection was reversed.

RESULTS

Adhesion of CHO Cells to the P21-Coated Plates—When CHO *pgsD*-677 cells were transfected with expression vector alone (mock), they showed a level of binding to the P21-coated plates similar to the non-treated *pgsD*-677 cells. However, those cells which had been transfected with CD9-cDNA exhibited a higher level of adhesion to the immobilized P21-peptide, comparable to that of CHO K1 cells (Fig. 1). This indicates that the CD9 antigen binds to the immobilized P21-peptide and that its site of interaction resides within its extracellular domains.

Inhibition of Adhesion of CD9-Expressing Cells to the Immobilized P21-Peptide by Heparin—The binding of CHO K1 cells, CHO *pgsD*-677 cells, and CD9-transfected CHO *pgsD*-677 cells to P21-coated plates was assayed in the presence of various concentrations of heparin (Fig. 2). The binding of CHO *pgsD*-677/CD9 cells to P21-coated plates was inhibited by heparin in a dose-dependent manner. At 0.0313 μg/ml, heparin depressed the binding of CHO *pgsD*-677/CD9 cells to the immobilized P21-peptide to the level of non-treated CHO *pgsD*-677 cells. The binding of CHO K1 cells to P21-coated plates was also examined. The extent of inhibition was weaker than that in the case of CHO *pgsD*-677 cells, but the binding was completely blocked when the concentration of heparin was increased to 2 μg/ml (data not shown).

Analysis of P21/Heparin Binding with SPR—In order to assess the non-specific binding of heparin to the CM-dextran matrix of the sensor chip, various concentrations of heparin were injected onto the negative control sensor chip (Fig. 3A). As shown, no binding of heparin to sensor chip matrix was observed. Overlaid sensorgrams of various concentrations of heparin to the immobilized P21-peptide are shown in Fig. 3B. The K_D of the heparin/P21-peptide interaction by steady-state analysis was calculated as: $K_D = (2.82 \pm 0.10) \times 10^{-8}$ M (mean $\pm$ SD, $n = 6$), assuming

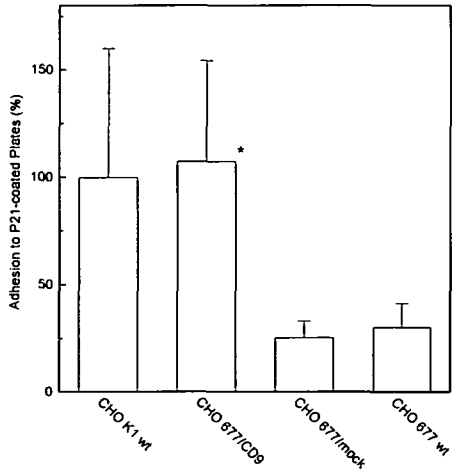

Fig. 1. **Adhesion of CHO cells to the P21-coated plates.** The number of cells (CHO K1 cells, CHO *pgsD*-677 cells [wt], CHO *pgsD*-677 cells transfected with expression vector only [mock], transfected with CD9 antigen cDNA) bound to the immobilized P21-peptide are shown. Binding levels are shown as a percentage relative to CHO K1 cells. Each result is the average of triplicate determinations and the SD is shown as error bars. $^*p < 0.05$ *vs.* CHO *pgsD*-677 cells [mock and wt] (Student's paired *t*-test).

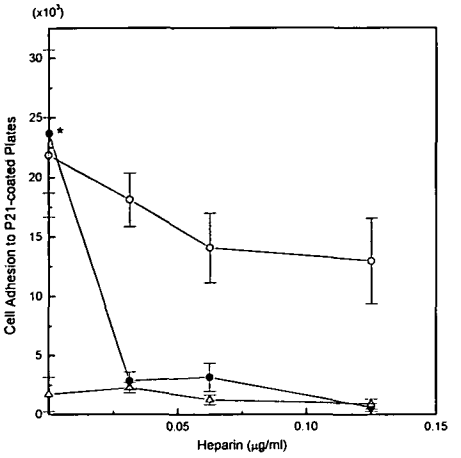

Fig. 2. **Inhibition of binding of CD9 antigen to the P21-peptide by heparin.** The binding levels of CHO K1 cells (○), CHO *pgsD*-677 cells transfected with CD9 antigen cDNA (●), and CHO *pgsD*-677 cells (△) to the P21-coated plates in the presence of various concentrations of heparin are shown. Each data point is the average of triplicate experiments. $^*p < 0.05$ *vs.* CHO *pgsD*-677 cells (Student's paired *t*-test).

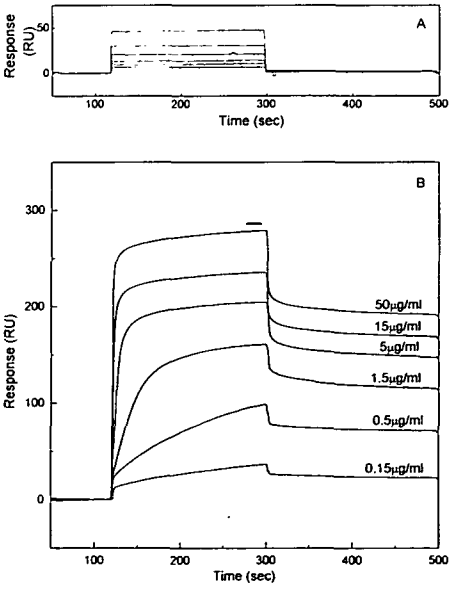

Fig. 3. **A: Binding of heparin to CM-dextran Sensor Chip matrix (negative control).** The sensorgrams of various concentrations of heparin were plotted together. Heparin was injected at 0.15, 0.5, 1.5, 5, 15, and 50 μg/ml, respectively. No binding of heparin to the Sensor Chip matrix itself was observed. **B: Binding of heparin to the immobilized P21-peptide.** The concentrations of heparin were the same as in A. The immobilization level of P21-peptide was 1,118.8 RU. The area used to calculate the kinetic constants is shown as a horizontal bar.

the molecular mass of heparin to be 19,000 Da. k_d and k_a values were calculated as $(2.72\pm0.15)\times10^{-4}$ s^{-1} and $(9.65\pm0.53)\times10^{3}$ $M^{-1}\cdot s^{-1}$, respectively.

Analyses of the P21-/CD9 Antigen Peptide Interaction with SPR—Solutions of eight synthetic peptides corresponding to the larger extracellular domain of CD9 antigen were injected onto immobilized/negative control flow cells, and their bindings were evaluated. In Fig. 4A, the sensorgrams of ^{109}AIWGYSHKDEVIKEVQEFYK^{128} peptide to the negative control flow cell (treated only with NHS/EDC/PDEA/cysteine) are shown as examples. No binding to the negative control flow cell was observed. The other seven peptides (Table I) showed no non-specific binding to the sensor chip matrix itself (data not shown). The sensorgram of the ^{109}A-K^{128} peptide to the immobilized P21-peptide is shown in Fig. 4B. For each concentration of peptide, the sensorgram to the negative control flow cell was subtracted from the corresponding sensorgram to the immobilized P21-peptide. No binding of ^{109}A-K^{128} peptide to P21-peptide was observed. The other six peptides, with the exception of ^{119}VIKEVQEFYKDTYNKLKTKD^{138}, displayed a similar sensorgram pattern.

Only the ^{119}V-D^{138} peptide showed specific binding to the immobilized P21-peptide. The resonance response increased as the concentration of this peptide was increased (Fig. 4C). As in panel B, the negative control sensorgrams for each concentration of the ^{119}V-D^{138} peptide were subtracted from those for the immobilized P21-peptide. The levels of the resonance signal were low, due to an weak p21-/^{119}V-D^{138} peptide interaction and/or low molecular weight of the injected ^{119}V-D^{138} peptide. However, dose-dependency of the resonance signal suggests that the binding between the ^{119}V-D^{138} and P21-peptide is specific. The K_D, k_d, and k_a values for this peptide/P21-peptide interaction were determined in a manner similar to those of P21/heparin: $K_D=(3.71\pm0.71)\times10^{-6}$ M, $k_d=(2.53\pm0.33)\times10^{-3}$ s^{-1}, and $k_a=(6.82\pm0.89)\times10^{2}$ $M^{-1}\cdot s^{-1}$, respectively (mean$\pm$SD, $n=6$).

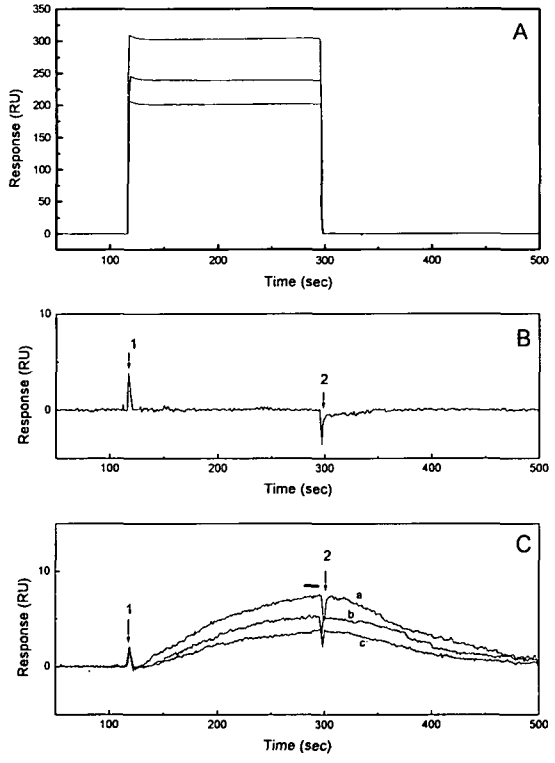

Fig. 4. **A: Binding of CD9 peptides to CM-dextran Sensor Chip matrix (negative control).** The time courses of the resonance signals of peptide ^{109}A-K^{128} are shown here as examples. The peptide was injected at 125, 250, and 500 μg/ml, respectively. No binding to the Sensor Chip matrix itself was observed. For the other seven synthetic peptides, corresponding to the larger extracellular domain of CD9 antigen, non-specific binding to the CM-dextran matrix was not observed (data not shown). **B: The binding of ^{109}AIWGYSHKDEVIKEVQEFYK^{128} peptide to the immobilized P21-peptide.** Peptide ^{109}A-K^{128} was injected at 120 s (arrow 1) and changed to running buffer at 300 s (arrow 2). The sensorgram of the blank run (negative control) was subtracted from the sensorgram to the immobilized P21-peptide. The peptide concentration was 500 μg/ml. **C: The binding ^{119}VIKEVQEFYKDTYNKLKTKD^{138} peptide to the immobilized P21-peptide.** The ^{119}V-D^{138} peptide was injected at 120 s (arrow 1) and then changed to running buffer at 300 s (arrow 2). The blank run sensorgrams (negative control) of each concentration of ^{119}V-D^{138} peptide were subtracted from each corresponding sensorgram to the immobilized P21-peptide. The peptide concentrations were 125 μg/ml (c), 250 μg/ml (b), and 500 μg/ml (a), respectively. The region used to analyze kinetic data is shown as a horizontal bar.

TABLE I. **Interaction between P21-peptide and peptides corresponding to the larger extracellular domain of CD9 antigen.** The numbers of amino acid residues are those of simian CD9 antigen (*12*).

Peptide	Binding with P21-peptide
^{109}AI WGYSHKDE VI KEVQEFYK^{128}	–
^{119}VI KEVQEFYK DTYNKLKTKD^{138}	+
^{129}DTYNKLKTKD EPQRETLKAI ^{148}	–
^{139}EPQRETLKAI HYALNCCGLA^{158}	–
^{149}HYALNCCGLA GGVEQFI SDI ^{168}	–
^{159}GGVEQFI SDI CPKKDVLETF^{178}	–
^{169}CPKKDVLETF TVKSCPDAI K^{188}	–
^{179}TVKSCPDAI K EVFDNKFI I G^{198}	–

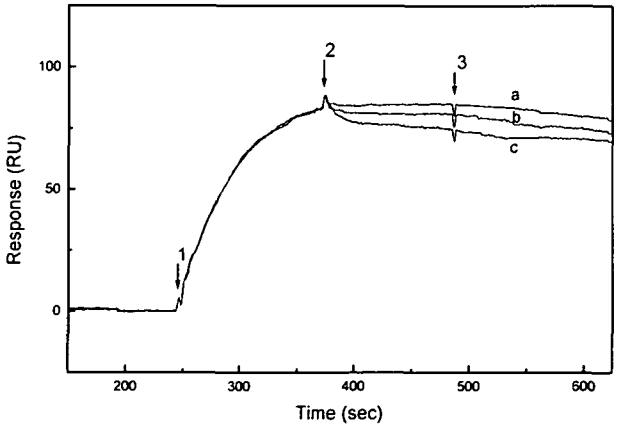

Fig. 5. **Overlaid sensorgrams for various concentrations of the ^{119}VIKEVQEFYKDTYNKLKTKD^{138} peptide injected sequentially after heparin.** The blank run sensorgrams (negative control) for each measurement were subtracted from the corresponding sensorgrams to the immobilized P21-peptide. The concentration of heparin for all sensorgrams was 0.5 μg/ml, and those of the ^{119}V-D^{138} peptide were 0 μg/ml (buffer only, c), 250 μg/ml (b), and 500 μg/ml (a), respectively. Heparin was injected at arrow 1, then exchanged to the ^{119}V-D^{138} peptide (arrow 2), and washed with running buffer (arrow 3).

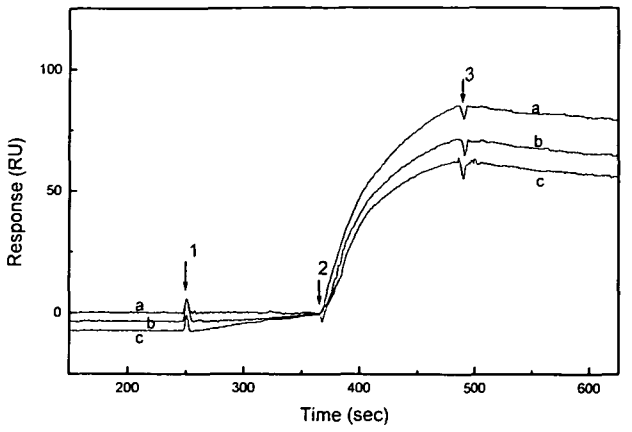

Fig. 6. **Results of sequential injection of the ^{119}VIKEVQEFYKDTYNKLKTKD^{138} peptide and heparin.** The blank run sensorgrams (negative control) for each measurement were subtracted from the corresponding sensorgrams to the immobilized P21-peptide. The sequence of injection was reversed from that in Fig. 5. The baseline was set to zero at the point of injection of heparin (arrow 2) to disclose any difference in the amount of binding of heparin. The concentrations of the ^{119}V-D^{138} peptide (injected at arrow 1) were 0 μg/ml (buffer only, a), 250 μg/ml (b), and 500 μg/ml (c), respectively, and the concentration of heparin was fixed at 0.5 μg/ml in all sensorgrams.

Sequential Injection of Heparin/CD9 ^{119}VIKEVQEFYKDTYNKLKTKD^{138} Peptide onto the Immobilized P21-Peptide—The ^{119}V-D^{138} peptide was sequentially injected after heparin onto the immobilized P21-peptide and the following resonance signal levels were monitored (Fig. 5). The signal levels increased with increasing the concentration of the ^{119}V-D^{138}. This suggests that the ^{119}V-D^{138} peptide also binds to the immobilized P21-peptide. When heparin was injected at 0.5 μg/ml ($\sim 2.6\times10^{-8}$ M), the immobilized P-21 peptide remained largely unoccupied (Fig. 3C). As a result, the ^{119}V-D^{138} peptide was able to bind to the immobilized P21-peptide and its response signals increased with increasing concentration.

When the injection order was reversed, the response signals of heparin decreased as the concentration of the pre-injected ^{119}V-D^{138} peptide was increased (Fig. 6). This suggests that the binding of heparin was hindered by the presence of the peptide ^{119}V-D^{138}, which had already bound to the immobilized P21-peptide. The molar concentration of the ^{119}V-D^{138} peptide was high (250 μg/ml [$\sim 1.0\times10^{-5}$ M] to 500 μg/ml [$\sim 2.0\times10^{-5}$ M]), compared to heparin, and the immobilized P21-peptide was considered to be largely occupied, although the resonance signal levels of the ^{119}V-D^{138} peptide were lower than heparin, because of its lower molecular weight.

When these two experiments are taken together, it can be concluded that both heparin and the peptide ^{119}V-D^{138} bind commonly to the P21-peptide.

DISCUSSION

While the interactions between HB-EGF and heparin/HSPG are analyzed in detail, the HB-EGF/CD9 antigen association is largely unresolved. However, the HBD of HB-EGF, which is located in its NH_2-terminus region, has been suggested to play an important role in the up-regulation of the juxtacrine activity of proHB-EGF mediated by CD9 antigen (*6*). Our results demonstrate that the extracellular domain of CD9 antigen binds to proHB-EGF through its HBD as follows: (1) CHO cells, which express CD9 antigen, bind to plates coated with the P21-peptide (Fig. 1); (2) the binding of CD9-expressing CHO cells to the immobilized P21-peptide is competitively inhibited by heparin in a dose-dependent manner (Fig. 2); (3) SPR analyses revealed that one of the peptides, which corresponds to the larger extracellular domain of CD9 antigen (^{119}VIKEVQEFYKDTYNKLKTKD^{138}), interacts with the immobilized P21-peptide (Fig. 3C, Table I); (4) the sequential injection of the ^{119}V-D^{138} peptide/heparin onto the SPR sensor chip, immobilized with the P21-peptide, showed that the HBD of HB-EGF represents the common binding site for CD9 antigen and heparin (Figs. 5 and 6).

CD9 antigen was initially identified as a 24-27 kDa protein in leukemia cells and developing B lymphocytes (*18, 19*). Platelets also express a large amount of CD9 antigen and can be activated by anti-CD9 antibodies (*20*). Moreover, CD9 antigen has also been detected in monocytes, endothelial cells, brain and peripheral nerves, vascular smooth muscle, and cardiac muscle (*21*). Although CD9 antigen has been known for some time, its specific physiological functions remain largely undetermined. However, its expression in a wide variety of cell lineage and the conservation of its amino acid sequence among a wide range of species [compared with green monkey CD9 antigen, 84.1% for cow (*22*), 89.0% for mouse (*23*), 90.7% for rat (*18*), and 99.1% for human (*17*)] strongly suggest that it plays a fundamental role in cellular functions.

SPR analysis yielded a K_D value for heparin/P21-peptide of $(2.82\pm0.10)\times10^{-8}$ M. However, heparin is a heterogeneous molecule with respect to molecular size. The molecular mass range of the heparin preparation used in this study was 5,000-30,000 Da, with an average molecular mass of 19,000 Da. Moreover, within a heparin molecule, the regions exist which are heavily sulfated (and hence more negatively charged), while other regions are not (*24*). Thus heparin is also heterogeneous with respect to its molecular composition. Furthermore, in SPR analyses, one reaction component is immobilized, and, therefore, does not necessarily represent the kinetic behavior of molecules in solution. The obtained K_D might well be an apparent value and caution should be exercised in interpreting the physiological meaning of this value.

The interaction of other growth factors, which possess heparin-binding properties, with heparin has also been studied. The K_D values of acidic and basic FGFs for low molecular weight heparin ($M_r \leq 6{,}000$) were estimated to be 91 nM and 2.0-3.1 nM, respectively (*26*), based on electrophoretic measurements. The K_D value of acidic FGF to 16-kDa heparin, as measured by SPR analysis, is 50-140 nM (*25*), while its K_D to low molecular weight heparin (average $M_r = 3{,}000$) determined with SPR was reported to be 500 nM (*27*). Therefore, the K_D values for heparin might vary with its preparation as well as the method of analysis.

Our results show that CD9 antigen binds to HB-EGF though its HBD and that the interaction sites of CD9 antigen resides within its larger extracellular domain, as judged by SPR techniques using synthetic peptides. Although both heparin/HSPG and CD9 antigen modulate

the functions of (pro)HB-EGF and bind to the same site of HB-EGF, there may be a difference in aspects of the functional regulation of HB-EGF. Whereas CD9 antigen is a membrane-bound molecule, heparin and HSPG exist in the extracellular matrix. Moreover, the K_D values for the P21-peptide/heparin and the P21-peptide/CD9 antigen-peptide (^{119}VIKEVQEFYKDTYNKLKTKD^{138}) interactions differed considerably: $K_D = (2.82 \pm 0.10) \times 10^{-8}$ M *vs.* $(3.71 \pm 0.71) \times 10^{-6}$ M. The binding of CHO *pgsD*-677/CD9 cells and CHO K1 cells to the P21-coated plates were inhibited in different manners by heparin. Whereas the binding of CHO *pgsD*-677/CD9 cells to the immobilized P21-peptide (mediated by CD9 antigen) was easily blocked with exogeneously added heparin (at 0.0313 μg/ml), that of CHO K1 cells (mediated by HSPG) was only partially inhibited at the same concentration of heparin (Fig. 2). This may be accounted for by the differences in the K_D values between the P21-peptide/heparin and the P21-/^{119}V-D^{138} peptide. Furthermore, this difference in affinity may be relevant to preferences for the regulation of (pro)HB-EGF *in vivo*. However, our results were obtained by means of an *in vitro* peptide-peptide and peptide-heparin interactions and may not be directly relevant to physiological conditions, where interactions between proteins and other molecules take place.

The interactions between peptide/peptide and peptide/protein that are involved in signal transduction have been analyzed with SPR. K_D, k_d, and k_a values between T-cell antigen receptor and the peptide bound to major histocompatibility complex are $(5.0\text{-}6.0) \times 10^{-5}$ M, $(5.7\text{-}9.0) \times 10^{-2}$ s^{-1}, and 9.0×10^2-1.7×10^3 $M^{-1} \cdot s^{-1}$, respectively (*28*). K_D value between the peptide corresponding to the Src homology 2 (SH2) domain of Src and the phosphopeptide from hamster polyoma middle T antigen is 0.67 μM and that between SH2 domain of p85 subunit of 1-phosphatidylinositol 3-kinase and the phosphopeptide from platelet-derived growth factor receptor is 0.24 μM, respectively (*29*). While the interaction between p21- and ^{119}V-D^{138} peptide is weaker than that of P21-peptide/heparin, it falls within the range of above results. The interaction of CD9 with HB-EGF may be significant in terms of signal transduction.

Although the binding of CD9-transfected cells to the P21-peptide indicates that the extracellular domain of CD9 antigen binds to the HBD of HB-EGF, it is possible that proHB-EGF and CD9 antigen interact with each other at the transmembrane domain or the intracellular domain as well. The probability that the smaller extracellular domain near N-terminus might also interact with HB-EGF can not be excluded. These need to be studied. Heparin/HSPG and CD9 antigen are known to modulate individually the function of (pro)HB-EGF. The mechanism by which they regulate (pro)HB-EGF remains to be clarified. The biology of the HB-EGF and its regulation by heparin/HSPG and CD9 antigen are clearly worthy of further study.

In conclusion, the interaction between CD9 antigen and proHB-EGF was studied. One of the binding sites of CD9 antigen to proHB-EGF was determined by means of SPR. This interaction was investigated in detail and K_D values were measured. Our present results might be useful in further studies of the regulation of the juxtacrine activity of proHB-EGF.

The authors greatly appreciate the technical suggestions of Mr. I. Okazaki and Ms. K. Morimoto (Pharmacia Biotech, Osaka).

REFERENCES

1. Higashiyama, S., Abraham, J.A., Miller, J., Fiddes, J.C., and Klagsbrun, M. (1991) A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* **251**, 936-939
2. Higashiyama, S., Abraham, J.A., and Klagsbrun, M. (1993) Heparin-binding EGF-like growth factor stimulation of smooth muscle cell migration: dependence on interactions with cell surface heparan sulfate. *J. Cell Biol.* **122**, 933-940
3. Thompson, S.A., Higashiyama, S., Wood, K., Pollitt, N.S., Damm, D., McEnroe, G., Garrick, B., Ashton, N., Lau, K., Hannock, N., Klagsbrun, M., and Abraham, J.A. (1994) Characterization of sequences within heparin-binding EGF-like growth factor that mediate interaction with heparin. *J. Biol. Chem.* **269**, 2541-2549
4. Naglich, J.G., Metherall, J.E., Russell, D.W., and Eidels, L. (1992) Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell* **69**, 1051-1061
5. Iwamoto, R., Higashiyama, S., Mitamura, T., Taniguchi, N., Klagsbrun, M., and Mekada, E. (1994) Heparin-binding EGF-like growth factor, which acts as the diphtheria toxin receptor, forms a complex with membrane protein DRAP27/CD9, which up-regulates functional receptors and diphtheria toxin sensitivity. *EMBO J.* **13**, 2322-2330
6. Higashiyama, S., Iwamoto, R., Goishi, K., Raab, G., Taniguchi, N., Klagsbrun, M., and Mekada, E. (1995) The membrane protein CD9/DRAP27 potentiates the juxtacrine growth factor activity of the membrane-anchored heparin-binding EGF-like growth factor. *J. Cell Biol.* **128**, 929-938
7. Coffey, R.J., Jr., Derynck, R., Wilcox, J.N., Bringman, T.S., Goustin, A.S., Moses, H.L., and Pittelkow, M.R. (1987) Production and auto-induction of transforming growth factor-α in human keratinocytes. *Nature* **328**, 817-820
8. Cook, P.W., Mattox, P.A., Keeble, W.W., Pittelkow, M.R., Plowman, G.D., Shoyab, M., Adelman, J.P., and Shipley, G.D. (1991) A heparin sulfate-regulated human keratinocyte autocrine factor is similar or identical to amphiregulin. *Mol. Cell. Biol.* **11**, 2547-2557
9. Hashimoto, K., Higashiyama, S., Asada, H., Hashimura, E., Kobayashi, T., Sudo, K., Nakagawa, T., Damm, D., Yoshikawa, K., and Taniguchi, N. (1994) Heparin-binding epidermal growth factor-like growth factor is an autocrine growth factor for human keratinocytes. *J. Biol. Chem.* **269**, 20060-20066
10. Inui, S., Higashiyama, S., Hashimoto, K., Higashiyama, M., Yoshikawa, K., and Taniguchi, N. (1997) Possible role of co-expression of CD9 with membrane-anchored heparin-binding EGF-like growth factor and amphiregulin in cultured human keratinocyte growth. *J. Cell. Physiol.* **171**, 291-298
11. Lidholt, K., Weinke, J.L., Kiser, C.S., Lugemwa, F.N., Bame, K.J., Cheifetz, S., Messagué, J., Lindahl, U., and Esko, J.D. (1992) A single mutation affects both *N*-acetylglucosaminyltransferase and glucuronosyltransferase activities in a Chinese hamster ovary cell mutant defective in heparan sulfate biosynthesis. *Proc. Natl. Acad. Sci. USA* **89**, 2267-2271
12. Mitamura, T., Iwamoto, R., Umata, T., Yomo, T., Urabe, I., Tsuneoka, M., and Mekada, E. (1992) The 27-kD diphtheria toxin receptor-associated protein (DRAP27) from Vero cells is the monkey homologue of human CD9 antigen: expression of DRAP27 elevates the number of diphtheria toxin receptors on toxin-sensitive cells. *J. Cell Biol.* **118**, 1389-1399
13. Johnsson, B., Löfås, S., and Lindquist, G. (1991) Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. *Anal. Biochem.* **198**, 268-277
14. Jönsson, U., Fägerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Löfås, S., Persson, B., Ross, H., Rönnberg, I., Sjölander, S., Stenberg, E., Ståhlberg, R, Urbaniczky, C., Östlin,

H., and Malmqvist, M. (1991) Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. *BioTechniques* **11**, 620-627
15. Karlsson, R. and Ståhlberg, R. (1995) Surface plasmon resonance detection and multispot sensing for direct monitoring of interactions involving low-molecular-weight analytes and for determination of low affinities. *Anal. Biochem.* **228**, 274-280
16. Boucheix, C., Benoit, P., Frachet, P., Billard, M., Worthington, R.E., Gagnon, J., and Uzan, G. (1991) Molecular cloning of the CD9 antigen. A new family of cell surface proteins. *J. Biol. Chem.* **266**, 117-122
17. Kaprielian, Z., Cho, K.-O., Hadjiargyrou, M., and Patterson, P.H. (1995) CD9, a major platelet cell surface glycoprotein, is a ROCA antigen and is expressed in the nervous system. *J. Neurosci.* **15**, 562-573
18. Kersey, J.H., LeBien, T.W., Abramson, C.S., Newman, R., Sutherland, R., and Greaves, M. (1981) p24: a human leukemia-associated and lymphohemopoietic progenitor cell surface structure identified with monoclonal antibody. *J. Exp. Med.* **153**, 726-731
19. Jones, N.H., Borowitz, M.J., and Metzgar, R.S. (1982) Characterization and distribution of a 24,000-molecular weight antigen defined by a monoclonal antibody (Du-All-1) elicited to common acute lymphoblastic leukemia (cALL) cells. *Leuk. Res.* **6**, 449-464
20. Boucheix, C., Soria, C., Mirshahi, M., Soria, J., Perrot, J.-Y., Fournier, N., Billard, M., and Rosenfeld, C. (1983) Characteristics of platelet aggregation induced by the monoclonal antibody ALB_6 (acute lymphoblastic leukemia antigen p24). Inhibition of aggregation by ALB_6Fab. *FEBS Lett.* **161**, 289-295
21. Jennings, L.K., Crossno, J.T., Jr., and White, M.M. (1995) CD9 cluster workshop report: cell surface binding and functional analysis in *Leucocyte Typing V* (Schlossman, S.F., Boumsell, L., Gilks, W., Harlan, J.M., Kishimoto, T., Morimoto, C., Ritz, J., Shaw, S., Silverstein, R., Springer, T., Tedder, T.F., and Todd, R.F., eds.) pp. 1249-1251, Oxford University Press, Oxford
22. Martín-Alonso, J.-M., Hernando, N., Ghosh, S., and Coca-Prados, M. (1992) Molecular cloning of the bovine CD9 antigen from ocular ciliary epithelial cells. *J. Biochem.* **112**, 63-67
23. Rubinstein, E., Billard, M., Plaisance, S., Prenant, M., and Boucheix, C. (1993) Molecular cloning of the mouse equivalent of CD9 antigen. *Thrombosis Res.* **71**, 377-383
24. Razi, N. and Lindahl, U. (1995) Biosynthesis of heparin/heparan sulfate. The D-glucosaminyl 3-*O*-sulfotransferase reaction: target and inhibitor saccharides. *J. Biol. Chem.* **270**, 11267-11275
25. Mach, H., Volkin, D.B., Burke, C.J., Middaugh, C.R., Linhardt, R.J., Fromm, J.R., Loganathan, D., and Mattson, L. (1993) Nature of the interaction of heparin with acidic fibroblast growth factor. *Biochemistry* **32**, 5480-5489
26. Lee, M.K. and Lander, A.D. (1991) Analysis of affinity and structural selectivity in the binding of proteins to glycosaminoglycans: development of a sensitive electrophoretic approach. *Proc. Natl. Acad. Sci. USA* **88**, 2768-2772
27. Lustig, F., Hoebeke, J., Östergren-Lundèn, G., Velge-Roussel, F., Bondjers, G., Olsson, U., Rüetschi, U., and Fager, G. (1996) Alternative splicing determines the binding of platelet-derived growth factor (PDGF-AA) to glycosaminoglycans. *Biochemistry* **35**, 12077-12085
28. Matsui, K., Boniface, J.J., Steffner, P., Reay, P.A., and Davis, M.M. (1994) Kinetics of T-cell receptor binding to peptide/I-E^k complexes: correlation of the dissociation rate with T-cell responsiveness. *Proc. Natl. Acad. Sci. USA* **91**, 12862-12866
29. Ladbury, J.E., Lemmon, M.A., Zhou, M., Green, J., Botfield, M.C., and Schlessinger, J. (1995) Measurement of the binding of tyrosyl phosphopeptides to SH2 domains: a reappraisal. *Proc. Natl. Acad. Sci. USA* **92**, 3199-3203