BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **242,** 197–201 (1998)
ARTICLE NO. RC977942

Epitope Mapping of Mouse Monoclonal Antibody EP-5C7 Which Neutralizes Both Human E- and P-selectin

Naoya Tsurushita,[1] Helen Fu, Jennifer Melrose, and Ellen L. Berg
Protein Design Labs, Inc., 2375 Garcia Avenue, Mountain View, California 94043

Received December 1, 1997

The epitope of mouse monoclonal antibody (mAb) EP-5C7, which binds to and blocks both human E- and P-selectin, was mapped onto the protein structure of E-selectin. Analyses with E- and L-selectin chimeric proteins and randomly mutagenized E-selectins demonstrated that the EP-5C7 epitope consists of the amino acid residues at positions 21, 22, 23, 119 and 120 of E-selectin. The binding of three neutralizing anti-E-selectin mAb's (E-1E4, H18/7 and CL2), whose epitopes were found to overlap with the E-selectin binding site for carbohydrate ligands, was not affected by the amino acid substitutions at these five positions. Inspection of the three-dimensional structure of E-selectin indicated that the EP-5C7 epitope is located near the junction between the lectin and EGF-like domains. The ligand binding site was distant from the EP-5C7 epitope, suggesting that the amino acid residues in the EP-5C7 epitope play an important role other than ligand binding in selectin-mediated cell adhesion. © 1998 Academic Press

Emigration of circulating leukocytes to inflammatory sites is known to be a multi-step process involving leukocyte rolling along activated endothelium followed by firm adhesion and transmigration (1,2). The selectin family of type I membrane glycoproteins mediates the initial adhesive interaction between leukocytes and endothelium (3). There are three selectins; L-selectin (CD62L), E-selectin (CD62E) and P-selectin (CD62P). L-selectin is expressed constitutively on most classes of leukocytes. E- and P-selectin are expressed on endothelial cells at sites of inflammation while P-selectin is also expressed on activated platelets. All three selectins are composed of an amino-terminal lectin-like domain, an epidermal growth factor (EGF)-like domain, a variable number of complement regulatory protein-like (CR) domains, a transmembrane domain and a short cytoplasmic domain. Selectins recognize sialyl-Lewis X or closely related carbohydrates expressed on specific proteins or glycolipids as their ligands in a Ca^{++}-dependent manner (4).

Recruitment of leukocytes at sites of inflammation is important for protection against infection and resolution of wounds; however, uncontrolled leukocyte recruitment results in tissue damage by neutrophils as observed in ischemia/reperfusion injury (5). In knockout mice lacking either E- or P-selectin, accumulation of neutrophils at sites of inflammation was only partially impaired (6,7). On the other hand, knockout mice lacking both E- and P-selectin showed severe defects in accumulation and extravasation of neutrophils (8). Therefore, it appears that E- and P-selectin are functionally redundant in neutrophil recruitment, and both must be blocked to optimally prevent neutrophils from causing tissue damage at sites of inflammation.

We previously reported the isolation of mouse mAb EP-5C7 which neutralizes the function of both human E- and P-selectin as adhesion molecules (9). Because of its dual reactivity, EP-5C7 is a promising drug candidate for treatment of inflammatory diseases caused by unregulated expression of E- and P-selectin. In order to better understand the mechanism of neutralization by EP-5C7, we identify here the EP-5C7 epitope at the amino acid level. The possible role of the EP-5C7 epitope in the function of E- and P-selectin is discussed.

MATERIALS AND METHODS

DNA manipulation. Plasmid pE/LE (Fig. 1) for the expression of truncated E-selectin was constructed as follows. The DNA fragments encoding the lectin and EGF-like domains (amino acid residues 1 to 158) were obtained by PCR using the E-selectin-encoding cDNA (10) as a template and appropriate primers. After digestion with *Nco*I and *Bam*HI, the DNA fragments were inserted between the *Nco*I and *Bam*HI sites of pNT3144, a derivative of the *E. coli* expression vector pScUAGΔcp3 (11), which carries the human Cλ gene (amino acid residues 109 to 215 according to Kabat et al.; 12).

Random mutagenesis in the lectin and EGF-like domains of E-selectin was performed by error-prone PCR (13) using NT150 and NT167 as primers and pE/LE as a template (Fig. 1). The PCR-amplified fragments were digested with *Nco*I and *Bam*HI, and inserted

[1] To whom correspondence should be addressed. Fax: 650-903-3730. E-mail: naoya@pdl.com.

0006-291X/98 $25.00

between the corresponding sites of pNT3144. Nucleotide substitutions were identified by DNA sequencing.

Cell lines. Chinese hamster ovary (CHO-K1) cells expressing truncated E-selectin ($CHO^{E\text{-}selectin}$), P-selectin ($CHO^{P\text{-}selectin}$), L-selectin ($CHO^{L\text{-}selectin}$) or chimeric E/L-selectin (CHO^{LEEE}, CHO^{ELLL}, CHO^{LELL} and CHO^{ELEE}) on the surface have been described (9,14). These CHO cell transfectants were maintained in glutamine-free DME medium supplemented with 10% dialyzed fetal calf serum (HyClone, Logan, UT).

Antibodies. Mouse mAb's EP-5C7 (anti-E-/P-selectin), E-1E4 (anti-E-selectin), WAPS12.2 (anti-P-selectin) and DREG-55 (anti-L-selectin) were described previously (9,15). Mouse anti-E-selectin mAb's H18/7 (16) and BBIG-E5 (17) were purchased from Beckton Dickinson (San Jose, CA) and R&D systems (Minneapolis, MN), respectively. Mouse anti-E-selectin mAb CL2 (18) was kindly provided by Dr. Eugene Butcher (Stanford University).

FACS and ELISA. FACS analysis of the binding of mAb to E-, P-, L- or chimeric E/L-selectins expressed on the surface of CHO cell transfectants was carried out as described (9,14) using a Becton-Dickinson FACScan.

Binding of mAb to truncated E-selectin fused to the λ chain constant region (E-selectin/Cλ) in *E. coli* culture supernatants was analyzed by sandwich ELISA. Wells of an ELISA plate were coated with 1 μg/ml test mAb. After blocking, IPTG-induced *E. coli* culture supernatants containing soluble E-selectin/Cλ fusion protein variants were added to the wells and incubated for 2 hr at room temperature. Bound E-selectin/Cλ were detected by incubation with peroxidase-conjugated goat anti-human λ chain antibodies (BioSource, Camarillo, CA) followed by color development with Turbo-TMB substrate (Pierce, Rockford, IL).

RESULTS

Chimeric E/L-Selectin

Mouse mAb EP-5C7 binds to both human E- and P-selectin and blocks cell-to-cell adhesion mediated by E- or P-selectin (9). To identify the epitope of EP-5C7, we first used CHO cell transfectants expressing truncated, cell-surface form of human E-selectin ($CHO^{E\text{-}selectin}$), P-selectin ($CHO^{P\text{-}selectin}$) or L-selectin ($CHO^{L\text{-}selectin}$) which contained the respective lectin, EGF-like, CR1 and CR2 domains. Two control mAb's, DREG-55 (anti-L-selectin) and WAPS12.2 (anti-P-selectin), showed expected binding patterns: DREG-55 bound only to $CHO^{L\text{-}selectin}$ and WAPS12.2 only to $CHO^{P\text{-}selectin}$ (Table I). EP-5C7 bound to $CHO^{E\text{-}selectin}$ and $CHO^{P\text{-}selectin}$, but not to $CHO^{L\text{-}selectin}$ (Table I), indicating that the EP-5C7 epitope lies within the lectin, EGF-like, CR1 and CR2 domains.

We then tested CHO cell transfectants expressing chimeric proteins containing the lectin, EGF-like, CR1 and CR2 domains of either human E- or L-selectin (E/L-selectin; Table I). Because EP-5C7 does not bind to L-selectin (9), E/L-selectins were used to determine the domain(s) of E-selectin necessary for binding of EP-5C7. In the control experiment, anti-P-selectin mAb WAPS12.2 did not bind to any of the four CHO cell transfectants expressing chimeric E/L-selectins (Table I). In addition, anti-L-selectin mAb DREG-55 bound to CHO^{LEEE} and CHO^{LELL}, but not to CHO^{ELLL} or CHO^{ELEE}, confirming the previous observation that the lectin domain of L-selectin is necessary for binding of DREG-55 (14). EP-5C7 bound to CHO^{ELLL} and CHO^{ELEE}, but not to CHO^{LEEE} or CHO^{LELL} (Table I). From this result, it is evident that the lectin domain of E-selectin is necessary for binding of EP-5C7.

We also analyzed the binding of four anti-E-selectin mAb's, E-1E4, H18/7, CL2 and BBIG-E5 (Table I). Three neutralizing mAb's, E-1E4, H18/7 and CL2, like EP-5C7, required the lectin domain of E-selectin for binding. On the other hand, the CR1 and CR2 domains of E-selectin were necessary for the binding of non-neutralizing mAb BBIG-E5.

E-Selectin Mutants

For further analysis of the EP-5C7 epitope, the DNA fragments encoding the lectin and EGF-like domains were cloned into an *E. coli* expression vector (pE/LE;

TABLE I

Binding of Monoclonal Antibodies to CHO Cell Transfectants Expressing Various Surface Selectins

		Binding of*[1]						
Cell line	Domains*[2]	EP-5C7	DREG-55	WAPS12/2	E-1E4	H18/7	CL2	BBIG-E5
$CHO^{E\text{-}selectin}$	EEEE	+	−	−	+	+	+	+
$CHO^{P\text{-}selectin}$	PPPP	+	−	+	−	−	−	−
$CHO^{L\text{-}selectin}$	LLLL	−	+	−	−	−	−	−
CHO^{ELLL}	ELLL	+	−	−	+	+	+	−
CHO^{LEEE}	LEEE	−	+	−	−	−	−	+
CHO^{ELEE}	ELEE	+	−	−	+	+	+	+
CHO^{LELL}	LELL	−	+	−	−	−	−	−

*[1] "+" indicates binding of test mAb to CHO cell transfectants. "−" indicates that the fluorescent signal was reduced to the background level.

*[2] The four letters represent, from the left, the lectin (amino acid 1 to 119), EGF-like (120 to 155), CR1 (156 to 217) and CR2 (218 to 282) domains of human E-selectin (E), P-selectin (P) or L-selectin (L). The CR2 domain of L-selectin contains the amino acid residues from 218 to 279.

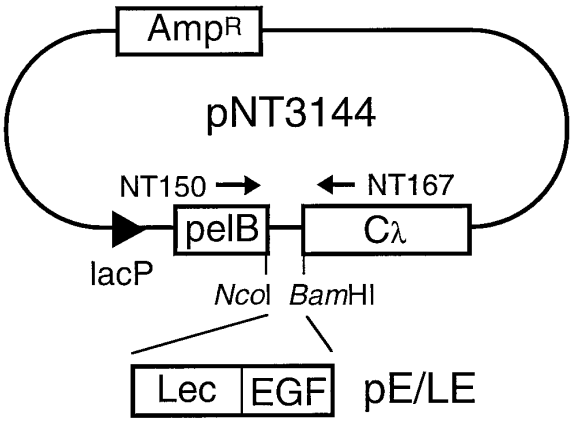

FIG. 1. Expression of truncated E-selectin in *E. coli*. Plasmid pNT3144 is a derivative of the *E. coli* expression vector pScUAGΔcp3 (11). Symbols used: Lec and EGF, the lectin and EGF-like domains of human E-selectin, respectively; lacP, *E. coli lac* promoter; pelB, *pel*B signal peptide; Cλ, the constant region of human λ light chain; Amp^R; *β*-lactamase gene for ampicillin resistance. Arrows show the positions and orientations of PCR primers, NT150 and NT167.

Fig. 1). By growing *E. coli* TG1ΔrecA carrying pE/LE in the presence of IPTG, truncated E-selectin fused to the human λ constant region (E-selectin/Cλ) was secreted in a soluble form into culture media (11). The binding of mAb to E-selectin/Cλ fusion proteins was analyzed by sandwich ELISA as described in Materials and Methods. Both anti-E-selectin mAb E-1E4 and anti-E-/P-selectin mAb EP-5C7 bound to E-selectin/Cλ.

To locate individual amino acid residues in E-selectin important for EP-5C7 binding, we introduced random amino acid substitutions in the lectin and EGF-like domains of E-selectin. The binding of E-selectin/Cλ mutants to EP-5C7 and E-1E4, which do not compete with each other in binding to E-selectin (9), was evaluated by sandwich ELISA (Table II). Among 33 single-amino-acid substitution mutants, binding of EP-5C7 to E-selectin/Cλ was abolished by the substitution of Gln at position 21 (counting from the amino terminus of the mature protein), Arg at 22, Tyr at 23, Thr at 119 or Ala at 120, while binding of E-1E4 was not affected by either of these five mutants (Table II). Therefore, these five amino acid residues should be located in or very close to the EP-5C7 epitope. Evidently, the EP-5C7 epitope is not a linear amino acid sequence.

Binding of E-1E4 to E-selectin/Cλ was completely abolished by the substitution of Tyr at 44, Tyr at 48, Glu at 92, Tyr at 94, Asn at 105, Lys at 111 or Leu at 114 (Table II). The binding of the other two neutralizing anti-E-selectin mAb, H18/7 and CL2, was abolished by the amino acid substitution at position 44 or 94, indicating that the epitopes of E-1E4, H18/7 and CL2 are overlapping.

Mapping of the EP-5C7 Epitope

To locate the EP-5C7 and E-1E4 epitopes on E-selectin, the amino acid residues affecting the binding of EP-5C7 or E-1E4 were mapped on a three-dimensional structure of the lectin and EGF-like domains of E-selectin. The amino acid residues necessary for binding of E-1E4 were located at the end of the lectin domain away from the junction with the EGF-like domain (Fig. 2B). Amino acid residues at positions 48, 82, 83, 92, 94, 97, 111 and 113 in the lectin domain of E-selectin are known to be important for neutrophil adhesion and have been implicated in ligand binding (19,20). These eight amino acid residues map to the same area as the E-1E4 epitope (Fig. 2C), suggesting that E-1E4, as well as H18/7 and CL2, which have epitopes overlapping the E-1E4 epitope, directly blocks the binding of E-selectin to carbohydrate ligands.

TABLE II

Binding of Monoclonal Antibodies to Mutant E-Selectins

	Binding*[2] of			
E-selectin*[1]	EP-5C7	E-1E4	H18/7	CL2
wild type	+	+	+	+
N4D	+	+	+	+
T7A	+	+	+	+
S16R	+	+	+/−	+/−
Q21R	−	+	+	+
R22G	−	+	+	+
Y23H	−	+	+	+
K32R	+	+	+	+
I35T	+	+	+	+
Y44H	+	−	−	−
Y48H	+	−	+	+
K55R	+	+	+	+
N57S	+	+	+	+
N58S	+	+	+	+
V61A	+	+	+	+
K67E	+	+	+	+
A73T	+	+	+	+
N83S	+	+/−	+	+
E92G	+	−	+	+
Y94H	+	−	−	−
R97G	+	+	+/−	+
V101A	+	+	+	+
N105S	+	−	+	+
S110G	+	+	+	+
K111E	+	−	+	+
K112R	+	+	+	+
L114H	+	−	+/−	+
T119A	−	+	+	+
A120T	−	+	+	+
N124S	+	+	+	+
T125A	+	+	+	+
S126A	+	+	+	+
L151H	+	+	+	+
N158D	+	+	+	+

*[1] The mutant name denotes the location of the amino acid substitution counting from the amino terminus of mature E-selectin (middle number), the wild-type amino acid residue (left letter; in single-letter code) and the amino acid residue in the mutant (right letter).

*[2] The symbols "+", "+/−" and "−" denote that the binding to mutant E-selectin/Cλ is more than 40%, 20 to 40%, and less than 20% of the binding to the wild-type, respectively.

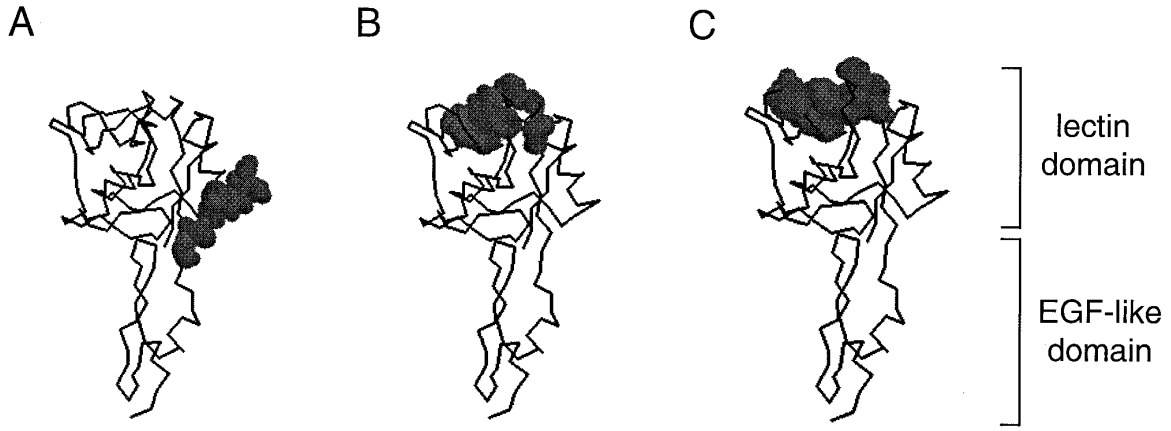

FIG. 2. Mapping of the EP-5C7 and E-1E4 epitopes on E-selectin. The three-dimensional structure of the lectin and EGF-like domains of human E-selectin was displayed with the RasMol molecular visualization program (R. Sayle, Glaxo Research and Development, Greenford, Middlesex, UK) based on the full atom coordinates by Graves et al. (1ESL, Brookhaven protein data bank; 20). *A,* EP-5C7 epitope. Gln at position 21, Arg at 22, Tyr at 23, Thr at 119 and Ala at 120 are shown in space-filling form. *B,* E-1E4 epitope. Tyr at 44, Tyr at 48, Glu at 92, Tyr at 94, Asn at 105, Lys at 111 and Leu at 114 are shown. *C,* Amino acid residues important for neutrophil binding. Tyr at 48, Asn at 82, Asn at 83, Glu at 92, Tyr at 94, Arg at 97, Lys at 111 and Lys at 113 are shown.

In contrast, the amino acid residues at positions 21, 22, 23, 119 and 120 necessary for binding of EP-5C7 were mapped to the side of the lectin domain near the junction with the EGF-like domain (Fig. 2A). The region occupied by the EP-5C7 epitope is distant from the carbohydrate binding site (Fig. 2C).

DISCUSSION

In this paper, we have demonstrated that the epitope of the dual-reactive anti-E-/P-selectin mAb EP-5C7 contains the amino acid residues at positions 20, 21, 22, 119 and 120 in E-selectin. Human E- and P-selectin share the same amino acid residues at positions 22, 23, 119 and 120; however, at position 21, E-selectin contains Gln while P-selectin contains Asn (Fig. 3). Such an amino acid change at position 21 appears not to affect the binding ability of EP-5C7 to E- and P-selectin significantly, probably because Gln and Asn are structurally similar to each other. Human L-selectin contains Asp at 21 and Asn at 22, which are different from the corresponding amino acid residues in E- or P-selectin (Fig. 3). This helps to explain why EP-5C7 does not bind to human L-selectin.

	18 26	116 123
E-selectin	YCQ**QRY**THL	.//.LCY**TA**ACT
P-selectin	---N---D-	-----S-Q
L-selectin	F-RDN--D-	-----S-Q

FIG. 3. Comparison of amino acid sequences surrounding the EP-5C7 epitope. Amino acid residues from position 18 to 26 and those from 116 to 123 of human E-selectin (23), human P-selectin (24) and human L-selectin (25) are shown in single-letter code. Amino acid residues of E-selectin at positions 21, 22, 23, 119 and 120 in the EP-5C7 epitope are shown in boldface. The symbol "-" denotes an amino acid residue identical to the counterpart of E-selectin.

How does EP-5C7 neutralize the function of E- and P-selectin? The results obtained in this work suggest that the binding of carbohydrate ligands is not affected by EP-5C7. First, the EP-5C7 epitope is distantly located from the carbohydrate binding site in the three-dimensional structure of E-selectin (Fig. 2A and C). Second, none of the amino acid substitutions in the EP-5C7 epitope affected the binding of neutralizing anti-E-selectin mAb, E-1E4, H18/7 and CL2, which bind to the carbohydrate binding site in the lectin domain (Table II). Third, the amino acid residues which interact with Ca^{++} ion necessary for ligand binding (Glu at 80, Asn at 82, Asn at 83, Asn at 105 and Asp at 106; 20) were mapped to be distant from the EP-5C7 epitope in the three-dimensional structure of E-selectin (data not shown). Because EP-5C7 is a potent neutralizing mAb, these results suggest that the amino acids residues in the EP-5C7 epitope are involved in an important step other than carbohydrate binding in the function of E- and P-selectin as adhesion molecules.
epitope in the function of P-selectin as an adhesion molecule.

Since the EP-5C7 epitope is located on the side of E-selectin, the amino acid residues in the EP-5C7 epitope might be involved in the selectin-selectin interaction to form multivalent selectin-ligand complex. The affinity between E-selectin and its carbohydrate ligand sialyl Lewis-X has been reported to be in the range of 0.1 to 1 mM (21). Although such a weak affinity may be beneficial in transient cell-to-cell interaction observed when leukocytes are rolling on endothelium, multivalent interaction between selectins and ligands appears to be required to retain leukocytes on endothelium even

transiently. In such a case, EP-5C7 would prevent the cluster formation of selectin-ligand complex, resulting in much weaker interaction between two cells. Alternatively, the EP-5C7 epitope might be involved in the interaction with other unknown molecules on the cell surface. Interestingly, the synthetic peptide composed of the amino acid residues from 16 to 30 of P-selectin, which contains a part of the EP-5C7 epitope (amino acid residues at 21, 22 and 23), was shown to inhibit neutrophils from binding to P-selectin, though the molecular mechanism of the inhibition is unknown (22). Further analyses of the amino acid residues in the EP-5C7 epitope could reveal an unidentified function of selectin in the molecular mechanism of selectin-mediated cell adhesion.

ACKNOWLEDGMENTS

We thank Mitchell Z. Ho, Jasmine Chow, Christine Fromm and Christine Johnson for technical assistance. We also thank Dr. Eugene Butcher for providing mAb WAPS12.2 and CL2, Dr. Max Vasquez for assisting in three-dimensional modeling and Dr. Cary Queen for his comments on the manuscript.

REFERENCES

1. Springer, T. A. (1994) *Cell* **76,** 301–314.
2. Carlos, T. M., and Harlan, J. M. (1994) *Blood* **84,** 2068–2101.
3. Kansas, G. S. (1996) *Blood* **88,** 3259–3287.
4. Varki, A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 7390–7397.
5. Lefer, A. M. (1995) *Ann. Thorac. Surg.* **60,** 773–777.
6. Labow, M. A., Norton, C. R., Rumberger, J. M., Lombard-Gillooly, K. M., Shuster, D. J., Hubbard, J., Bertko, R., Knaack, P. A., Terry, R. W., Harbison, M. L., Kontgen, F., Stewart, C. L., McIntyre, K. W., Will, P. C., Burns, D. K., and Wolitzky, B. A. (1994) *Immunity* **1,** 709–720.
7. Mayadas, T. N., Johnson, R. C., Rayburn, H., Hynes, R. O., and Wagner, D. D. (1993) *Cell* **74,** 541–554.
8. Frenette, P. S., Mayadas, T. N., Rayburn, H., Hynes, R. O., and Wagner, D. D. (1996) *Cell* **84,** 563–574.
9. Berg, E. L., Fromm, C., Melrose, J., and Tsurushita, N. (1995) *Blood* **85,** 31–37.
10. Berg, E. L., Robinson, M. K., Mansson, O., Butcher, E. C., and Magnani, J. L. (1991) *J. Biol. Chem.* **266,** 14869–14872.
11. Akamatsu, Y., Cole, M. S., Tso, J. Y., and Tsurushita, N. (1993) *J. Immunol.* **151,** 4651–4659.
12. Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S., and Foeller, C. (1991) Sequences of Proteins of Immunological Interest, 5th ed., U.S. Dept. Health Human Services, Washington, DC.
13. Leung, D. W., Chen, E., and Goeddel, D. V. (1989) *Technique* **1,** 11–15.
14. Fu, H., Berg, E. L., and Tsurushita, N. (1997) *Immunol. Lett.* **59,** 71–77.
15. Kishimoto, T. K., Jutlia, M. A., and Butcher, E. C. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 2244–2248.
16. Bevilacqua, M. P., Pober, J. S., Mendrick, D. L., Cotran, R. S., and Gimbrone Jr., A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 9238–9242.
17. Pigott, R., Needham, L. A., Edwards, M. R., Walker, C., and Power, C. (1991) *J. Immunol.* **147,** 130–135.
18. Kishimoto, T. K., Warnock, R. A., Jutila, M. A., Butcher, E. C., Lane, C., Anderson, D. C., and Smith, C. W. (1991) *Blood* **78,** 805–811.
19. Erbe, D. V., Wolitzky, B. A., Presta, L. G., Norton, C. R., Ramos, R. J., Burns, D. K., Rumberger, J. M., Rao, B. N., Foxall, C., Brandley, B. K., and Lasky, L. A. (1992) *J. Cell Biol.* **119,** 215–227.
20. Graves, B. J., Crowther, R. L., Chandran, C., Rumberger, J. M., Li, S., Huang, K.-S., Presky, D. H., Familletti, P. C., Wolitzky, B. A., and Burns, D. K. (1994) *Nature* **367,** 532–538.
21. Jacob, G. S., Kirmaier, C., Abbas, S. Z., Howard, S. C., Steininger, C. N., Welply, J. K., and Scudder, P. (1995) *Biochemistry* **34,** 1210–1217.
22. Heavner, G. A., Falcone, M., Kruszynski, M., Epps, L., Mervic, M., Riexinger, D., and McEver, R. P. (1993) *Int. J. Peptide Protein Res.* **42,** 484–489.
23. Hession, C., Osborn, L., Goff, D., Chi-Rosso, G., Vassallo, C., Pasek, M., Pittack, C., Tizard, R., Goelz, S., McCarthy, K., Hopple, S., and Lobb, R. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1673–1677.
24. Johnston, G. I., Cook, R. G., and McEver, R. P. (1989) *Cell* **56,** 1033–1044.
25. Camerini, D., James, S. P., Stamenkovic, I., and Seed, B. (1989) *Nature* **342,** 78–82.