Location of the domains of ICAM-1 by immunolabeling and single-molecule electron microscopy

Tomas Kirchhausen,* Donald E. Staunton,† and Timothy A. Springer†

*Center for Blood Research, *Department of Anatomy and Cellular Biology, and †Department of Pathology, Harvard Medical School, Boston, Massachusetts*

Abstract: Intercellular adhesion molecule 1 (ICAM-1), a member of the immunoglobulin gene superfamily, is a cell surface glycoprotein with an extracellular domain comprising five immunoglobulin-like domains. Soluble ICAM-1, a recombinant protein truncated at the transmembrane domain, has a rod-like shape, 19 nm long overall, with a characteristic bend 7.6 nm from one end of the molecule. Because the link between domain D2 and domain D3 is proline rich, it has been proposed that the short arm contains domains D1 and D2 and the long arm contains domains D3–D5. We used single-molecule electron microscopy of soluble ICAM-1 decorated with monoclonal antibodies specific for domains D1 and D4 to show that the bend instead lies between domains D3 and D4. Therefore, the short arm lies closer to the plasma membrane, whereas the long arm, containing all the known ligand binding sites on ICAM-1, is positioned toward the target cell surface. *J. Leukoc. Biol.* 53: 342–346; 1993.

Key Words: *structure • adhesion receptors • epitope mapping • ICAM-1*

INTRODUCTION

Intercellular adhesion molecule 1 (ICAM-1, CD54) is a cell surface protein that promotes leukocyte adhesion during immune and inflammatory responses by interactions with the integrins lymphocyte function–associated antigen 1 (LFA-1) and Mac-1 [1–3]. ICAM-1 is, in addition, a cellular receptor for rhinoviruses [4, 5] and a sequestration receptor for erythrocytes infected with the malaria parasite *Plasmodium falciparum* [6]. ICAM-1 is a single glycosylated polypeptide chain of molecular weight ~ 90,000 composed of five tandem immunoglobulin (Ig)-like domains located at the cell surface and linked by a transmembrane segment to a small intracellular carboxyl terminal domain. The amino-terminal Ig-like domain (D1) of ICAM-1 is involved in the interaction with LFA-1, rhinoviruses, and *P. falciparum*–infected erythrocytes [7–9]. The third Ig-like domain (D3) of ICAM-1 contains the binding site for the integrin Mac-1 [3], and the function of the other three domains is unknown.

A previous electron microscopic study of recombinant sICAM-1, a soluble fragment corresponding to the five Ig-like domains of ICAM-1, has shown rod-shaped images of uniform thickness ~ 19 nm in length with a characteristic bend 7.6 nm from one end [7]. The position of this bend defines a short and a long arm presumed to contain two and three Ig-like domains, respectively. Based on the relatively high content of proline residues in the link between the second (D2) and third (D3) Ig-like domains and the presence of prolines in Ig hinge regions, it was proposed that the bend might be located between D2 and D3 so that D1 and D2 form the short arm and D3, D4, and D5 the long arm [7].

We used monoclonal antibodies to decorate specific domains in ICAM-1. By visualizing the complexes using single-molecule electron microscopy, we show that the bend lies between D3 and D4 instead of D2 and D3, as previously hypothesized. The images reveal that an epitope located in D1 of ICAM-1 maps to the distal end of the long arm and that an epitope located in D4 maps to a site near or at the bend. The electron microscopic observations also confirm the previous suggestion that the five Ig-like domains of ICAM-1 are unpaired.

MATERIALS AND METHODS

Preparation of soluble ICAM-1 and its monoclonal antibodies

A soluble form of human ICAM-1 with all five Ig-like domains (sICAM-1) was generated by oligonucleotide-directed mutagenesis [7] and purified from culture media of Chinese hamster ovary (CHO) cell transfectants by immunoaffinity chromatography as described previously [10]. Purified ICAM-1 mouse monoclonal antibodies CL203 [2, 11] and LB2 [12] were a gift from Dr. Steve Marlin (CL203) and Dr. Edward Clark (LB2).

Formation of soluble ICAM-1–antibody complexes

To generate immunocomplexes, sICAM-1 (12 μl at ~ 0.1 mg/ml) was mixed with equimolar amounts of the relevant mouse monoclonal antibody (1 μl at ~ 0.5 mg/ml) and incubated for 1 h at 4°C in 13.7 mM NaCl, 0.27 mM KCl, 1 mM Na_2HPO_4, 0.18 mM KH_2PO_4 (0.1 × phosphate-buffered saline). At the end of this period, 6 μl of the mixture was rapidly diluted with 45 μl of cold 40% glycerol followed by spray and platinum (Pt) shadowing.

Abbreviations: CHO, Chinese hamster ovary; ICAM-1, intercellular adhesion molecule 1; Ig, immunoglobulin; LFA-1, lymphocyte function–associated antigen 1.

Reprint requests: Tomas Kirchhausen, Center for Blood Research and Department of Anatomy and Cellular Biology, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115.

Received September 4, 1992; accepted November 22, 1992.

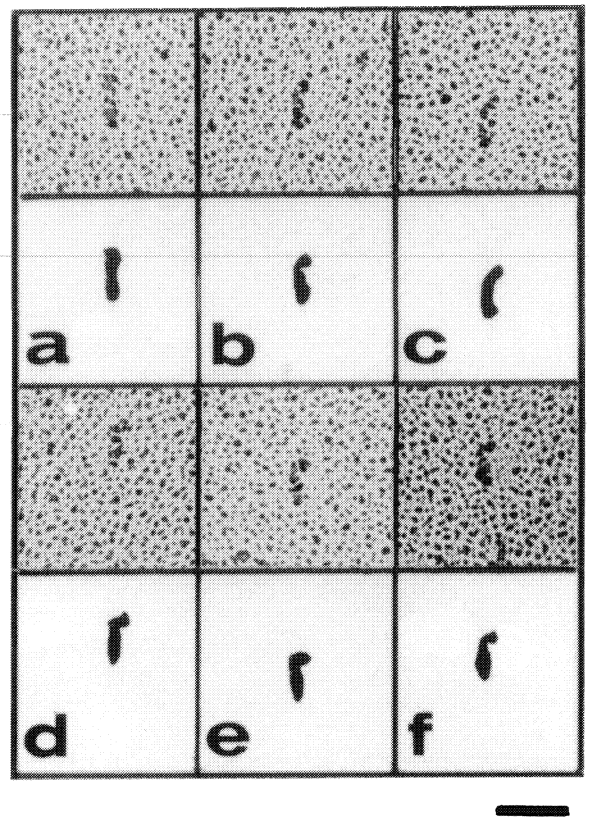

Fig. 1. Glycerol-sprayed and Pt-shadowed images of human sICAM-1. The gallery of selected images shows sICAM-1 with its extended conformation (a) and with its characteristic bend (b–f). Bar, 25 nm.

Electron microscopy and visualization of ICAM-1–antibody complexes

Single molecules of sICAM-1 complexed to its antibodies were visualized by electron microscopy by glycerol spray and Pt shadowing [13]. Samples were sprayed onto freshly cleaved mica, dried under vacuum, and rotary shadowed with Pt at an angle of 7–8°. Electron micrographs of single molecules were obtained with a primary magnification of 36,000–48,000 at 80 kV in a JEOL 100 CXII electron microscope. The pictures were printed so that the molecules lie between the viewer and the mica surface.

The set of images used to assign the localization of domains for the extracellular portion of ICAM-1 includes only those views in which both the outline of sICAM-1 and the point of contact with the antibody were visible. Images containing sICAM-1 molecules with foreshortened outlines or ill-defined bends were not used in the analysis. These pictures may correspond to oblique or top views of the immunocomplexes and in principle could be analyzed by image reconstruction. However, the Pt grains used for shadowing are large (2–3 nm) relative to the imaged molecules and therefore the error involved in subtracting the size of these grains, to determine the shape of the molecules, is potentially large. Furthermore, it is unclear whether the bend in ICAM-1 and the joint between ICAM-1 and the bound antibody are rigid or flexible, which would also complicate the analysis.

RESULTS AND DISCUSSION

The electron microscopic visualization experiments described here were performed with human sICAM-1, a fragment of 453 amino acids corresponding to the extracellular domain of ICAM-1 purified from the media of transfected CHO cells. Shown in **Figure 1** is a gallery of selected images obtained from a Pt rotary shadowed preparation of pure sICAM-1. The pictures confirm previous results of Staunton et al. [7] showing that sICAM-1 appears as a homogeneous population of single rods about 18–19 nm long by 2–3 nm thick, frequently displaying a characteristic bend located about two-fifths along its length.

We have relied on the asymmetric shape of sICAM-1, as defined by the location of its bend, to orient its short and long arms with respect to its five Ig-like domains. We visualized two classes of immunocomplexes of sICAM-1, one generated by incubation with the monoclonal antibody LB2, directed against an epitope in D1 of ICAM-1 [7] (**Fig. 2b**), the other by incubation with the monoclonal antibody CL203, directed against an epitope in D4 [7] (Fig. 2a). These pictures, and others not shown, contain views that can be assigned to immunocomplexes of sICAM-1 and its antibodies (circles). The consistency of the images and the absence of prominent aggregates made by sICAM-1 or by antibodies alone exclude the possibility that the visualized complexes were generated by random collisions between sICAM-1 and its antibodies. These images were interpreted as views of globular antibodies bound to unique epitopes along the rod-shaped sICAM-1, under the assumption that molecular contacts are specific and that the visualized complexes are relatively undistorted by the experimental procedure. The remaining images had shapes characteristic of free sICAM-1 (arrowhead) or free monoclonal antibody (asterisk).

The general field in Figure 2a and the gallery of selected images in **Figure 3** correspond to immunocomplexes formed by recognition of the epitope in D4 of sICAM-1 by the monoclonal antibody CL203. The most prominent and frequent type of view, representing ~ 50% of identified complexes (from a total of 100 views scored in three independent fields), shows the outline of an sICAM-1 molecule lying sideways to the antibody (Fig. 3a–g). In these views, the angle of the bend in sICAM-1 is in close contact with the antibody, whereas the rod portions of the long and short arms of sICAM-1 extend away from the globular antibody. Our interpretation of these images is that D4 of ICAM-1 maps to a location close to the bend of sICAM-1. A related type of view, found in ~ 22% of the images, contained two sICAM-1 molecules cross-linked by a single CL203 antibody (Fig. 3h–l); the point of contact in each case is at the bend in sICAM-1. The interpretation of the remaining 15% of the images was more complex. For example, some of the images showed only a straight rod, presumably the long arm of sICAM-1, projecting away from CL203. This type of view could be generated if the immunocomplex adsorbed to the mica oriented in such a way that the short arm of sICAM-1 lay above (or below) its long arm or that the antibody covered one of the arms. Other images were views of sICAM-1 without its bend, lying sideways to the an-

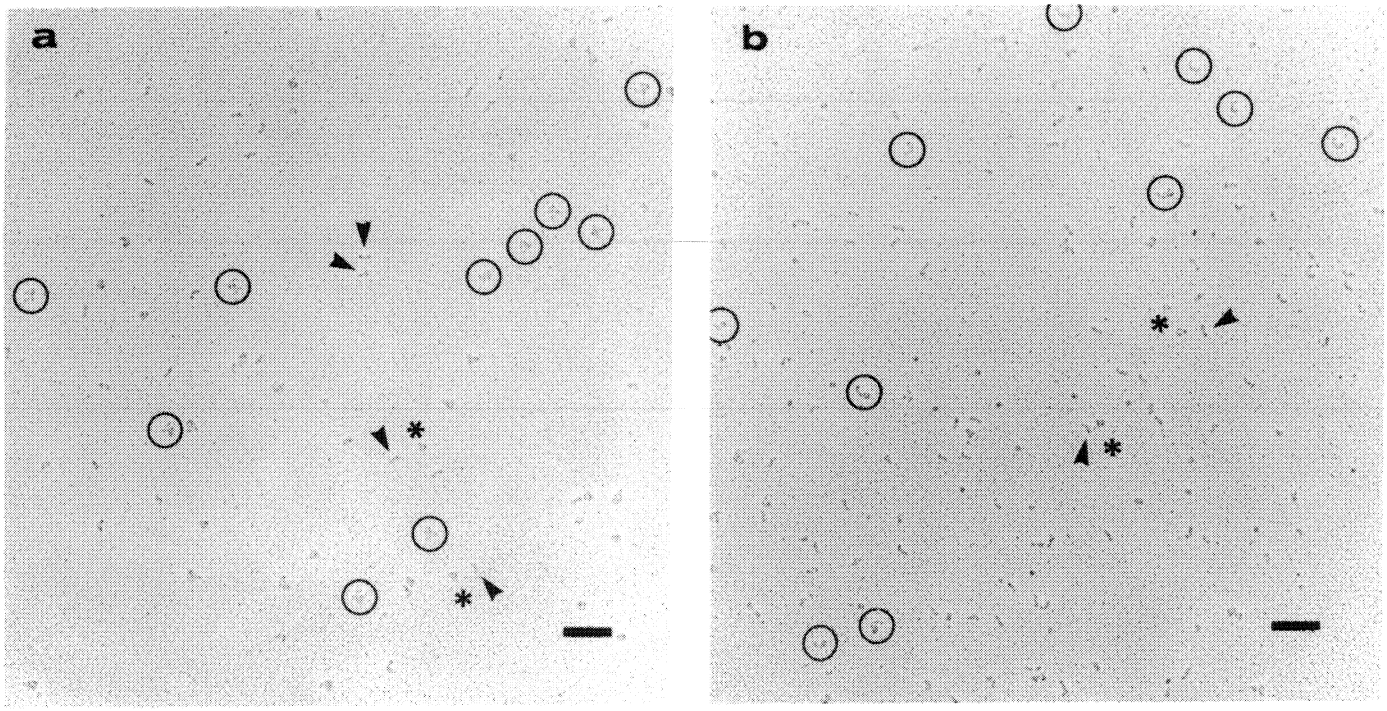

Fig. 2. Association of human sICAM-1 with monoclonal antibodies CL203 and LB2. Solutions containing human sICAM-1 and the mouse monoclonal antibodies CL203 and LB2 were prepared as described in Materials and Methods, glycerol sprayed, and visualized by Pt shadowing. Examples of interpretable images of immunocomplexes of sICAM-1 with its antibody are circled. Free sICAM-1 molecules (arrowhead) and IgG antibodies (asterisk) are indicated. (a) sICAM-1 with antibody CL203; (b) sICAM-1 with antibody LB2. Bar, 100 nm.

tibody. Finally, 2% of the images showed an antibody positioned at the end of the short arm of sICAM-1.

In contrast, the monoclonal antibody LB2, which recognizes an epitope in D4, binds to a site in sICAM-1 located at the distal end of its long arm. Figure 2b and **Figure 4** correspond to a field and to selected images of immunocomplexes obtained by the interaction of this antibody with sICAM-1. Most images of recognizable and well-defined complexes can be described as a single globular antibody bound to an end of the rod-shaped sICAM-1. In about 54% of these images (from a total of 70 views scored; Fig. 4a–l), the short arm of sICAM-1 lies away from the antibody and displays a clockwise orientation. The apparent handedness of these images reflects a preferential attachment to the mica from one side of the immunocomplex, probably due to its surface charge distribution. The same handedness is seen with free sICAM-1 (Fig. 1) and its complexes with monoclonal antibody CL203 (Fig. 2a and 3), suggesting that the bend is not as flexible as previously assumed. In the remaining images

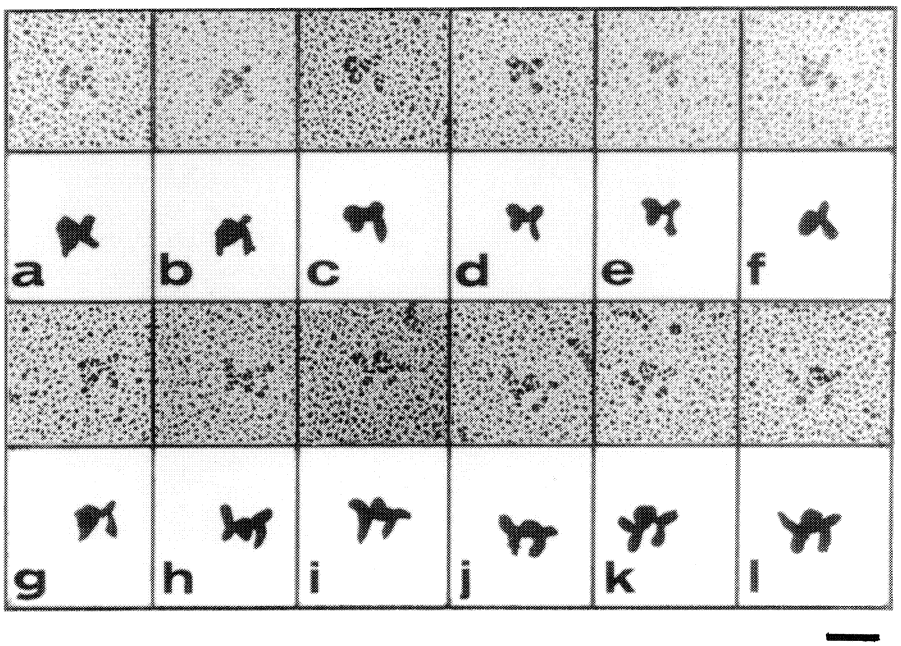

Fig. 3. Selected views of human sICAM-1 complexed to the antibody CL203. The Pt-shadowed images and their interpretations included in this gallery were chosen because they clearly demonstrate the characteristic bend of sICAM-1 and the globular outline of the antibody. The epitope recognized by this antibody is located in domain D4 of ICAM-1; (a–g) one sICAM-1 molecule bound to CL203, (h–l) two sICAM-1 molecules bound to CL203. Bar, 25 nm.

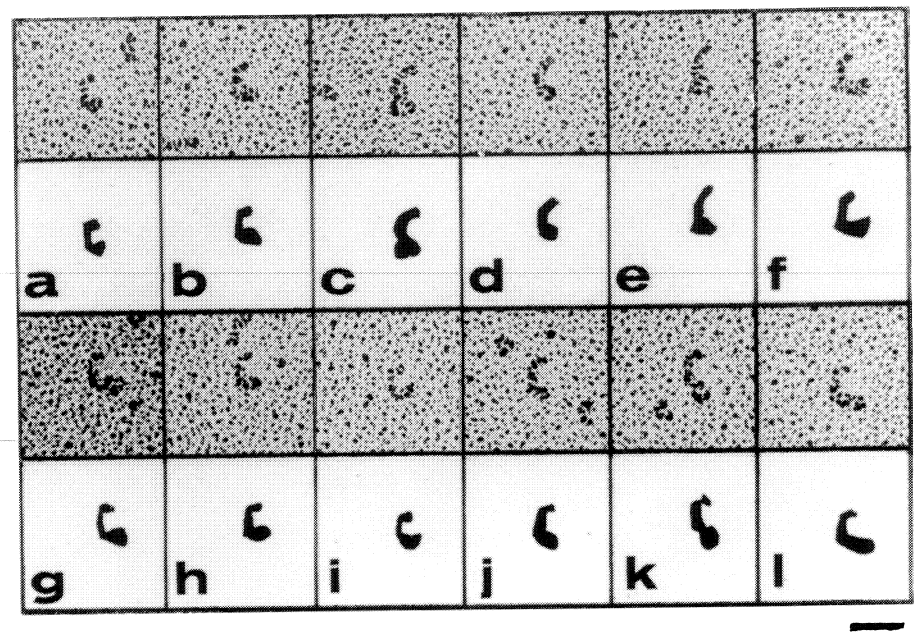

Fig. 4. Selected views of human sICAM-1 complexed to antibody LB2. The epitope recognized by this antibody is located in domain D1 of ICAM-1. These images show the antibody bound at the distal end of the long arm of sICAM-1. Bar, 25 nm.

(not shown), the bend was absent and the short arm was not well defined, presumably representing foreshortened views of sICAM-1. Since the epitope recognized by LB2 is located at the amino-terminal domain (D1) of ICAM-1, we propose that this domain is located at the distal end of the long arm. Consistent with this interpretation, we found that in most of the remaining views the end of a straight sICAM-1 was bound to the antibody.

The schematic drawing in **Figure 5** summarizes our model of ICAM-1. This representation derives from the interpretation of the electron micrographs of human sICAM-1 complexed to the mouse monoclonal antibodies CL203 and LB2.

The two antibodies we have studied bind selectively to opposite ends of the rod-shaped sICAM-1. Based on the shape and dimensions of sICAM-1, it was previously proposed that its five Ig-like domains are collinear with the length of the rod of ICAM-1. Our results confirm this model because the two antibodies we have studied, which recognize epitopes in domains D1 and D4, bind selectively to opposite ends of the rod-shaped sICAM-1. Moreover, we have established for the first time the absolute configuration of ICAM-1 by locating D1 at the distal end of the long arm and D4 in the vicinity of the bend in sICAM-1. Because the carboxyl-terminal end of ICAM-1 lies inside the cell, this arrangement positions the bend in the Ig-like extracellular domains toward the proximity of the plasma membrane. This conformation would result in a significant area of the surface of the ICAM-1 molecule lying parallel to neighboring cells and available for interaction. Furthermore, rotational freedom of ICAM-1 around its transmembrane segment would facilitate, in addition to lateral diffusion of the transmembrane segment, movement on the cell surface of the long axis of ICAM-1 with domains 1–3. Indeed, this arrangement is consistent with the observation that the binding sites for integrins LFA-1 and Mac-1 are in D1 and D3, the first and third Ig-like domains of ICAM-1 [3, 7].

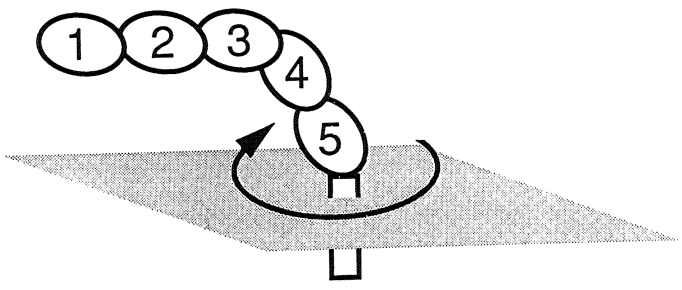

Fig. 5. Schematic representation of ICAM-1. The location of domains with respect to the bent rod axis is based on the results of single-molecule electron microscopy.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (T.K.) and CA31798 (T.A.S.). We thank C. Montes for secretarial assistance and K. Dayton for photographic help.

REFERENCES

1. Springer, T.A., Dustin, M.L., Kishimoto, T.K., Marlin, S.D. (1987) The lymphocyte function–associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. 1. *Annu. Rev. Immunol.* **5,** 223–252.
2. Springer, T.A. (1990) Adhesion receptors of the immune system. *Nature* **346,** 425–434.
3. Diamond, M.S., Staunton, D.E., Marlin, S.D., Springer, T.A. (1991) Binding of the integrin Mac-1 (CD11b/CD18) to the third Ig-like domain of ICAM-1 (CD54) and its regulation by glycosylation. *Cell* **65,** 961–971.
4. Greve, J.M., Davis, G., Meyer, A.M., Forte, C.P., Yost, S.C., Marlor, C.W., Kamarck, M.E., McClelland, A. (1989) The major human rhinovirus receptor is ICAM-1. *Cell* **56,** 839–847.
5. Staunton, D.E., Merluzzi, V.J., Rothlein, R., Barton, R., Marlin, S.D., Springer, T.A. (1989) A cell adhesion molecule,

ICAM-1, is the major surface receptor for rhinoviruses. *Cell* **56,** 849–853.
6. Berendt, A.R., Simmons, D.L., Tansey, J., Newbold, C.E., Marsh, K. (1989) Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum*. *Nature* **341,** 57–59.
7. Staunton, D.E., Dustin, M.L., Erickson, H.P., Springer, T.A. (1990) The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. *Cell* **61,** 243–254.
8. Ockenhouse, C.F., Betageri, R., Springer, T.A., Staunton, D.E. (1992) *Plasmodium falciparum*–infected erythrocytes bind ICAM-1 at a site distinct from LFA-1, Mac-1, and human rhinovirus. *Cell* **68,** 63–69.
9. Berendt, A.R., McDowall, A., Craig, A.G., Bates, P.A., Sternberg, M.J.E., Marsh, K., Newbold, C.I., Hogg, N. (1992) The binding site on ICAM-1 for *Plasmodium falciparum*–infected erythrocytes overlaps, but is distinct from, the LFA-1-binding site. *Cell* **68,** 71–81.
10. Marlin, S.D., Staunton, D.E., Springer, T.A., Stratowa, C., Sommergruber, W., Merluzzi, V. (1990) A soluble form of intercellular adhesion molecule-1 inhibits rhinovirus infection. *Nature* **344,** 70–72.
11. Matsui, M., Temponi, M., Ferrone, S. (1987) Characterization of a monoclonal antibody–defined human melanoma–associated antigen susceptible to induction by immune interferon. *J. Immunol.* **139,** 2088–2095.
12. Clark, E.A., Ledbetter, J.A., Holly, R.C., Dinndorf, P.A., Shu, G. (1986) Polypeptides on human B lymphocytes associated with cell activation. *Hum. Immunol.* **16,** 100–113.
13. Fowler, W.E., Erickson, H.P. (1979) Trinodular structure of fibrinogen. Confirmation by both shadowing and negative stain-electron microscopy. *J. Mol. Biol.* **134,** 241–249.