separate from those which bind its natural ligands, then this would present new possibilities for AIDS therapy.

We would like to thank Ms Yvonne Schneemann for excellent technical assistance, Ms Judie Hossmann for preparing the manuscript, and Drs Alan Tunnacliffe and Eddy Roosnek for critical reading. We would also like to thank Dr H. Kiefer for CD4-specific oligonucleotide preparation, Dr S. Ryser for providing human genomic libraries and Dr J. Mous for helpful discussions. Purified gp120 was a gift from Dr P. Berman and the anti-CD4 monoclonal antibodies were provided by Dr R. van Lier. The Basel Institute for Immunology was founded and is supported by F. Hoffman-La Roche, Ltd Co., Basel, Switzerland.

Received 9 November; accepted 13 November 1987.

1. Curran, J. *et al. Science* 229, 1352-1357 (1985).
2. Weiss, R. *et al. Nature* 324, 572-575 (1986).
3. Dalgleish, A. *et al. Nature* 312, 763-766 (1984).
4. Klatzmann, D. & Gluckman, J. C. *Immunol. Today* 7, 291-297 (1986).
5. McDougal, ... *et al. J. Immun.* 135, 3151-3162 (1985).
6. Reinherz, E. L. & Schlossman, S. F. *Cell* 19, 821-827 (1980).
7. Klatzmann, D. *et al. Nature* 312, 767-768 (1984).
8. McDougal, J. *et al. J. Immun.* 137, 2937-2944 (1986).
9. Maddon, P. J. *et al. Cell* 47, 333-348 (1986).
10. Lasky, L. A. *et al. Cell* 50, 975-985 (1987).
11. Starcich, B. R. *et al. Cell* 45, 637-648 (1986).
12. Alizon, M., Wain-Hobson, S., Montagnier, L. & Sonigo, P. *Cell* 46, 63-74 (1986).
13. Willey, R. *et al. Proc. Natn. Acad. Sci. U.S.A.* 83, 5038-5042 (1986)
14. *Hahn, B. et al. Science* 232, 1548-1553 (1986).
15. Maddon, P. J. *et al. Cell* 42, 93-104 (1985).
16. Littman, D. R. & Gettner, S. N. *Nature* 325, 453-455 (1987).
17. Traunecker, A., Dolder, B. & Karjalainen, K. *Eur. J. Immun.* 16, 851-854 (1986).
18. Classon, J. B., Tsagarotos, J., McKenzie, I. F. & Walker, I. D. *Proc. natn. Acad. Sci. U.S.A.* 83, 4499-4503 (1986).
19. Harada, S., Purtilo, D. T., Koyanagi, Y., Sonnabend, J. & Yamamoto, N. J. *immun. Meth.* 92, 177-181 (1986).
20. Harada, S., Yoshiyama, H. & Yamamoto, N. *J. Clin. Microbiol.* 22, 908-911 (1985).
21. Harada, S., Koyonagi, Y. & Yamamoto, N. *Science* 229, 563-566 (1985).

ICAM-1 a ligand for LFA-1-dependent adhesion of B, T and myeloid cells

Malegapuru W. Makgoba*‖, Martin E. Sanders*, Gale E. Ginther Luce*, Michael L. Dustin†, Timothy A. Springer†, Edward A. Clark‡, Patrice Mannoni§ & Stephen Shaw*

* Immunology Branch, NCI, NIH, Bethesda, Maryland 20892, USA
† Dana Farber Cancer Institute, Boston, Massachusetts 02115, USA
‡ Regional Primate Research Center, Washington University, Seattle, Washington 98195, USA
§ Inserm Unit 119, 27 Boulevard Lei Roure, Marseille 13009, France

Cell–cell adhesion is essential for many immunological functions[1–4]. The LFA-1 molecule, a member of a superfamily of adhesion molecules, participates in adhesion which is critical to the function of each of the three major subsets of leukocytes: lymphocytes, monocytes and granulocytes[5]. Putative LFA-1 ligands have been identified functionally in different laboratories using three different monoclonal antibodies that inhibit LFA-1-mediated leukocyte adhesion in particular model systems; however, there may be more than one LFA-1 ligand[6–8]. We have directly compared the three relevant monoclonal antibodies, and show that each binds to the same molecule, intercellular-adhesion molecule-1 (ICAM-1). Most important, B, T and myeloid cells adhere specifically to purified ICAM-1-coated surfaces; such adhesion has distinctive requirements for Mg^{2+} and Ca^{2+}. This constitutes biochemical evidence that ICAM-1 functions as a ligand for LFA-1-dependent adhesion by a variety of leukocytes.

Recent functional studies indicate that ICAM-1, recognized by the monoclonal antibody (mAb) RR1/1, is a ligand for LFA-1 in several models of antigen-independent B- and T-cell adhesion and in cytotoxic T-cell recognition[6–9]. Several other mAbs have recently been identified with properties suggesting that they also define molecules which function as ligands for LFA-1 in adhesion by different kinds of leukocytes. These include: (1) LB-2, a B-cell activation marker[10] which inhibits both B- and myeloid homotypic-cell adhesion[11]; and (2) 84H10, which was identified by screening for preferential binding to myeloid leukaemic cells and subsequently shown to inhibit the adhesion of such cells to bone-marrow stromal cells (P.M., unpublished data). Furthermore 84H10 has been shown to inhibit homotypic-B-cell aggregation[12]. Here we use three different approaches which show that these three mAbs bind a single molecule (ICAM-1) and that purified ICAM-1 functions as a ligand for LFA-1 in cell adhesion.

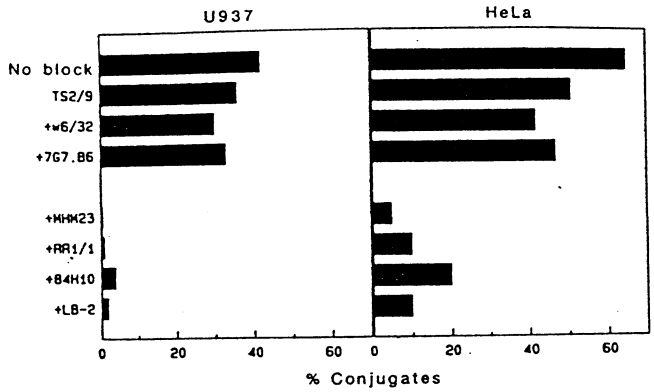

Fig. 1 Inhibition of LFA-1-dependent conjugate formation. Conjugate formation was measured between the cytotoxic T-cell clone 8.2 and two antigen-negative targets, U937 and HeLa. Conjugates were enumerated by two-colour cytometry as described[15,24]. To isolate the LFA-1 pathway of adhesion the LFA-3 mAb TS2/9 was continuously present in all assays (except the 'no block') in saturating concentrations to inhibit adhesion via the CD2/LFA-3 pathway[15,24]. Antibodies used were LFA-3 mAb TS2/9 (ref. 25), HLA class I mAb w6/32 (ref. 26), IL-2 mAb 7G7.B (ref. 27), LFA-1β mAb MHM23 (ref. 28), RR1/1, 84H10 and LB-2. Antibodies were continuously present during the assay at 100 $\mu g\ ml^{-1}$ purified IgG except for MHM23 and TS2/9 which were present as 300 $\mu g\ ml^{-1}$ of Fab fragment.

We compared the ability of the three mAbs to inhibit LFA-1-dependent conjugate formation between a cytotoxic T-cell clone and two antigen-negative targets of different lineage: a myeloid line (U937) and a cervical-carcinoma line (HeLa) (Fig. 1). Each of the putative ICAM-1 antibodies inhibited LFA-1-dependent conjugate formation with each target cell but control mAbs did not. The residual conjugate formation between the T-cell clone and HeLa cells in the presence of each putative ICAM-1 mAbs may reflect the existence of an additional LFA-1 ligand on HeLa cells as previously proposed[6,8].

To determine whether LB-2, 84H10 and RR1/1 mAbs bind the same molecule, each was assayed for its ability to bind the molecule immunoaffinity-purified on an 84H10 column (Fig. 2). All three antibodies bound the purified 84H10 antigen even at concentrations of 5ng ml^{-1}; the specificity of binding was shown by lack of binding by irrelevant LFA-3 mAb. Results of competitive-binding studies indicate that RR1/1 binds to an epitope that is spatially separate from that to which 84H10 and LB-2 bind (data not shown). Thus, RR1/1, LB-2 and 84H10 bind to epitopes on the same molecule, which we call ICAM-1 as proposed by Rothlein *et al.*[6]. This finding unifies observations from multiple laboratories regarding the function, structure, tissue distribution and genetics of ICAM-1. Most important, functional understanding of ICAM-1 is broadened by correlating the data reported for RR1/1, LB-2 and 84H10, and by our studies using purified ICAM-1 (see below). RR1/1 detects a single chain varying in its relative molecular mass (M_r) from 90 to 114K[6], LB-2 detects a chain of 76K[10] or 84K[11] and 84H10 to a chain of 105K[13]. This variation is consistent with results

‖ Present address: Department of Chemical Pathology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0HS, UK.

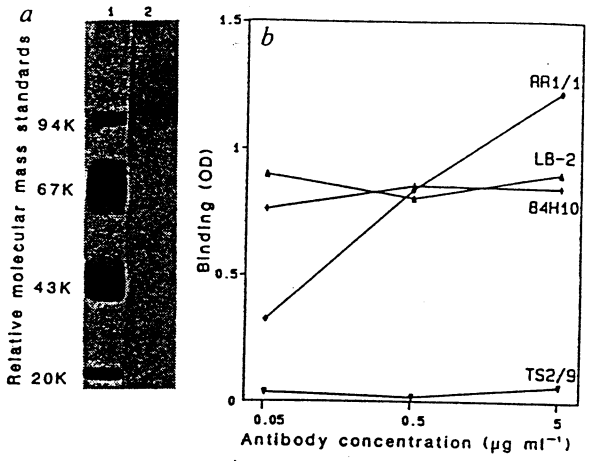

Fig. 2 Binding of three mAb to purified ICAM-1. Triton X-100 solubilized material from 3×10^8 cells of the L428 Reed Sternberg line [illegible] was purified on a column of 84H10 mAb coupled to Sepharose-4B beads. After washing the column, material was eluted at *p*H 2.5 in 0.01 M Tris/0.05 M glycine buffer. The eluate ran as a single band of 95–97K on a 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) (*a*, lane 2). *a*, Lane 1 shows relative molecular-mass standards. Purified ICAM-1 was applied to glutaraldehyde/poly-L-lysine-treated microtitre plates as follows: 96-well Nunc-Immunoplates were treated with 100 μl per well of a 0.2% glutaraldehyde in 0.1 M sodium carbonate-HCl *p*H 9 for 1 h at room temperature. The plate was washed twice with distilled water. 50 μl of poly-L-lysine (50 μg per ml in 0.05 M sodium bicarbonate) were added to each well and left to incubate for 2 h. The plate was washed twice with 0.05 M sodium bicarbonate. To each well 25 μl of purified ICAM-1 and 25 μl of 0.2% glutaraldehyde (in 0.05 M sodium bicarbonate) were added. Incubation was allowed to proceed for 8 h. Unbound sites were neutralized by filling each well with 0.05 M Tris-HCl *p*H 7.8 plus 2.5% human serum albumin (HSA) for 2 h. The plate was washed twice with Tris-HSA. Antibody binding to the immobilized antigen *b*) was measured by enzyme-linked immunosorbent assay (ELISA) using purified mAb (IgG) as the first step, β-galactosidase-conjugated $F(ab')_2$ sheep anti-mouse IgG (heavy plus light chains) as the second step, and measuring the rate of conversion of *p*-nitrophenyl-β-galactoside to a coloured product (using Bethesda Research Laboratories hybridoma screening reagent). Data are given as the average of the optical density at 405 nm of duplicate wells after subtraction of the OD in the absence of a first-step antibody; standard errors were less than 10% of the mean. No specific binding was observed in control wells, in which antibodies were incubated in HSA-coated wells (data not shown).

showing that ICAM-1 is synthesized from a precursor of 73K, of which 55K is protein, and that cell-type-dependent differences in carbohydrate processing account for variation in M_r. Staining shows that ICAM-1 is present in a variety of cells in tissue sections, particularly endothelium[6,9]; a pattern consistent with the staining pattern of 84H10 (ref. 13). Our analysis of staining on a variety of *in vitro* cell lines shows complete concordance in binding of the antibodies RR1/1, LB-2 and 84H10 (M.W.M., in preparation). Enhancement of antibody binding following cell activation is characteristic not only of LB-2 (ref. 11), but also of RR1/1 (ref. 9) and 84H10 (data not shown). The gene coding for ICAM-1 maps to chromosome 19, based on previous studies with the LB-2 mAb[10].

Inhibition of LFA-1-dependent binding by ICAM-1 mAb RR1/1 at the target level[8,9] has been the basis for the inference that ICAM-1 is a ligand for LFA-1. But alternative explanations are possible, for example that binding of ICAM-1 mAb delivers an inhibitory signal which prevents target participation in LFA-1-dependent adhesion. To test directly the hypothesis that ICAM-1 is a ligand involved in LFA-1-dependent adhesion, we studied cell adhesion to purified ICAM-1 (Fig. 3). LFA-1-positive cells of three different lineages (a lymphoblastoid B cell, a cytotoxic T-cell clone and a promonocytoid cell) bound to

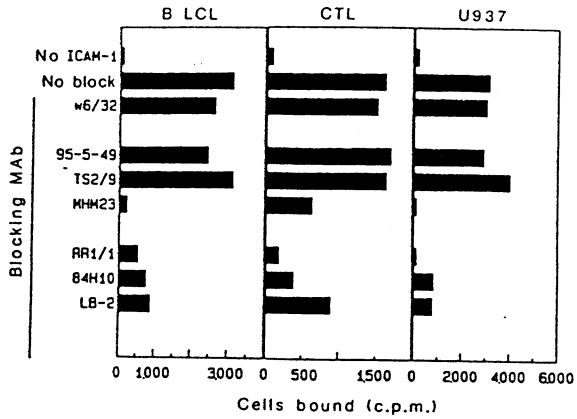

Fig. 3 Antibody inhibition of cell binding to purified ICAM-1 immobilized on microtitre plates. Plates were prepared as described in the legend to Fig. 2. Control wells (designated 'no ICAM-1') were coated with HSA; all other wells were coated with purified ICAM-1. Cells of three different leukocyte lineages were labelled with ^{51}Cr and 10^5 cells added to each well in 100 μl of phosphate-buffered saline (PBS) 0.5% HSA, and incubated for 20 min at 37 °C. Antibodies being tested for their ability to inhibit cell binding were present continuously at 50 μg ml^{-1} final concentration. The plates were washed gently three times with PBS/0.5% HSA to remove unbound cells. The bound cells were lysed with 5% Triton X-100 and the lysate counted for radioactivity. Results are expressed as the mean c.p.m. of duplicate wells; standard errors are all less than 15% of the mean. Antibodies used were as described in Fig. 1 legend, and CD2 mAb 95-5-49 (ref. 30).

ICAM-1 immobilized on plastic. Three lines of evidence show that this adhesion is specific: (1) the adhesion is inhibited by the three ICAM-1 mAbs and the LFA-1 mAbs but not by the LFA-3, CD2 and class I histocompatibility locus antigen (HLA) mAbs; (2) the cells did not adhere to other proteins immobilized on plastic (for instance albumin, Fig. 3); (3) cells like L428 that lack LFA-1 expression did not bind to plastic-immobilized ICAM-1 (data not shown). These results confirm and extend studies using purified ICAM-1 from a RR1/1 affinity column, incorporated into phospholipid membranes, (S.D.M. and T.A.S. *Cell*, in press). These studies show directly that ICAM-1 is a ligand for LFA-1-dependent cell adhesion. The simplest interpretation of these data is that ICAM-1 is a ligand for LFA-1. However, the possibility that ICAM-1 is a ligand for some other adhesion receptor whose function depends on LFA-1 cannot be excluded.

LFA-1-dependent adhesion has been shown previously to require divalent cations[14,15]. We analysed the cation requirements of LFA-1-dependent adhesion to purified ICAM-1 and directly compared them with the cation requirements of intercellular adhesion via the molecules LFA-1 and ICAM-1 (Fig. 4). The similarities between the results of the two assay systems are striking: Mg^{2+} alone is sufficient for optimal adhesion; Ca^{2+} alone is not, but can synergize with suboptimal concentrations of Mg^{2+}. For example, 1 mM Ca^{2+} supports minimal (<10% of optimal) adhesion in each system, however, in the presence of 0.1 mM Mg^{2+}, 1 mM Ca^{2+} enhances adhesion from ~30% to ~90% of the maximal adhesion. The similarities between the two assay systems emphasize that the present assay of cell binding to purified ICAM-1 faithfully reflects the biology of LFA-1 interactions which are involved in more complex intercellular interactions. This pattern of cation requirement is characteristic of LFA-1-dependent adhesion and not observed, for example, with adhesion of the same cells via CD2/LFA-3 interaction[15]. These data indicate that divalent cations are required for the interaction of LFA-1 with ICAM-1. Two divalent-cation-binding sites may be involved: one requiring $Mg^{(2+)}$, which is saturated at Mg^{2+} concentrations below 0.1 mM, and another which binds either Mg^{2+} or Ca^{2+} but with somewhat lower affinity than the first site.

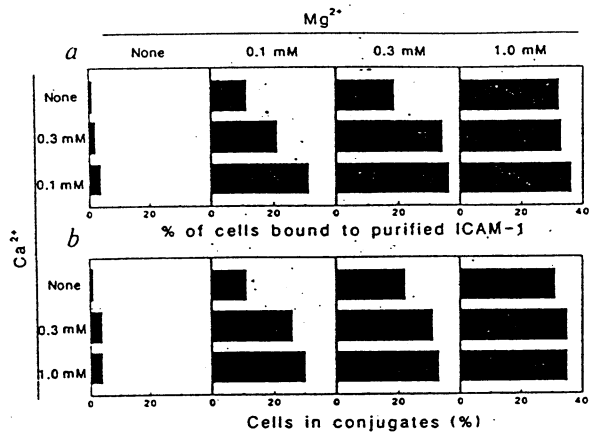

Fig. 4 Distinctive divalent-cation requirements of LFA-1-dependent adhesion to ICAM-1. The ability of Ca^{2+} and Mg^{2+} to support cell binding to purified ICAM-1 (*a*) was compared with the requirement for Ca^{2+} and Mg^{2+} in intercellular adhesion (*b*). Binding to ICAM-1 was measured as outlined in Fig. 3 and T-cell conjugates as described in Fig. 1 except that the buffer used in both assays was HEPES-buffered saline consisting of 0.13 M NaCl, 10 mM HEPES, 2 mg ml^{-1} glucose, 5 mg ml^{-1} HSA. $MgCl_2$ and $CaCl_2$ were added at the concentrations indicated during the assay. Cells used were B LCL in panel *a* and T-cell clone 8.2 with target U937 in panel *b*; U937 was chosen as the target cell because it uses ICAM-1 almost exclusively as a ligand in LFA-1-dependent intercellular adhesion (see also Fig. 1).

Multiple lines of evidence show that ICAM-1 is a ligand for LFA-1-dependent adhesion of multiple lineages of haematopoietic cells: T cells, B cells and myeloid cells bind to purified ICAM-1 (Fig. 3); RR1/1 inhibits binding of T, B and myeloid cells[6,9]; LB-2 mAb inhibits adhesion of B and myeloid cells[11]; 84H10 inhibits binding of myeloid cells (P.M., unpublished) and B cells[12]. In normal tissue sections, staining is most intense on endothelium but is increased on many cells by such factors as inflammation, exposure to cytokines and neoplastic transformation[5]. Thus, the role of ICAM-1 as an LFA-1 ligand may be critical in immune surveillance during inflammation or neoplastic transformation.

Structural information on LFA-1 shows that it is part of a large family of cell-interaction molecules highly conserved throughout evolution. LFA-1 and the other molecules with which it shares a β chain (MAC-1 and p150/95) are restricted in their distribution to haematopoietic cells[5] but they are closely related to more widely-distributed families of mammalian adhesion molecules and even to the *Drosophila* position-specific protein which is critical for cell positioning during embryogenesis and morphogenesis[16-22]. It remains to be shown whether ICAM-1, like other ligands for many of these adhesion receptors, has a critical RGD peptide in its sequence and whether it can be used as a ligand by more than one receptor.

T.A.S. is supported by the NIH. We thank Drs R. E. Gress, J. E. K. Hildreth, W. E. Biddison and R. Quinones for mAbs and Drs W. E. Biddison and P. Henkart for useful discussion.

Received 11 August; accepted 6 November 1987.

1. Rouse, R. V., Reichert, R. A., Gallatin, W. M., Weissman, I. L. & Butcher, E. C. *Am. J. Anat.* 170, 391-405 (1984).
2. Bjerknes, M., Cheng, H. & Ottaway, C. A. *Science* 231, 402-405 (1986).
3. Bell, G. I. *Immun. Today* 4, 237-240 (1983).
4. Inaba, K. & Steinman, R. M. *J. exp. Med.* 163, 247-261 (1986).
5. Springer, T. A., Dustin, M. L., Kishimoto, T. K. & Marlin, S. D. *A. Rev. Immun.* 5, 223-252 (1987).
6. Rothlein, R., Dustin, M. L., Marlin, S. D. & Springer, T. A. *J. Immun.* 137, 1270-1274 (1986).
7. Makgoba, M. W. *et al. Ann. N.Y. Acad. Sci.* (in the press).
8. Makgoba, M. W. *et al. Eur. J. Immun.* (in the press).
9. Dustin, M. L., Rothlein, R., Bhan, A. K., Dinarello, C. A. & Springer, T. A. *J. Immun.* 137, 245-254 (1986).
10. Clark, E. A., Ledbetter, J. A., Holly, R. C., Dinndorf, P. A. & Shu, G. *Hum. Immun.* 16, 100-113 (1986).
11. Patarroyo, M., Clark, E. A., Prieto, J., Kantor, C. & Gahmberg, C. G. *FEBS Lett.* 210, 127-131 (1987).
12. Marlin, S. D., Miller, L. M. & Springer, T. A. in *Leucocyte typing III. White cell differentiation antigens* (eds McMichael, A. J. *et al.*) 832-833 (Oxford University Press, New York, 1987).
13. Hogg, N. & Horton, M. A. in *Leucocyte Typing III, White Cell Differentiation Antigens* (eds McMichael, A. J. *et al.*) 576-602 (Oxford University Press, Oxford, 1987).
14. Martz, E. *J. Cell Biol.* 84, 584-598 (1980).
15. Shaw, S. *et al. Nature* 323, 262-264 (1986).
16. Hynes, R. O. *Cell* 48, 549-554 (1987).
17. Lawler, J. & Hynes, R. O. *J. Cell Biol.* 103, 1635-1648 (1986).
18. Suzuki, S. *et al. Proc. natn. Acad. Sci. U.S.A.* 83, 8614-8618 (1986).
19. Takada, Y., Strominger, J. L. & Hemler, M. E. *Proc. natn. Acad. Sci. U.S.A.* 84, 3239-3243 (1987).
20. Law, S. K. A. *et al. EMBO J.* 6, 915-919 (1987).
21. Miller, L. J., Wiebe, M. & Springer, T. A. *J. Immun.* 138, 2381-2383 (1987).
22. Kishimoto, T. K., O'Connor, K., Lee, A., Roberts, T. M. & Springer, T. A. *Cell* 48, 681-690 (1987).
23. Luce, G. G., Gallop, P. M., Sharrow, S. O. & Shaw, S. *BioTechniques* 3, 270-272 (1985).
24. Shaw, S. & Luce, G. G. *J. Immun.* 139, 1037-1045 (1987).
25. Sanchez-Madrid, F. *et al. Proc. natn. Acad. Sci. U.S.A.* 79, 7489-7493 (1982).
26. Barnstable, C. J. *et al. Cell* 14, 9-20 (1978).
27. Rubin, L. A., Kurman, C. C., Biddison, W. E., Goldman, N. D. & Nelson, D. L. *Hybridoma* 4, 91-102 (1985).
28. Hildreth, J. E. & August, J. T. *J. Immun.* 134, 3272-3280 (1985).
29. Schaadt, M., Diehl, V., Stein, H., Fonatsch, C. & Kirchner, H. H. *Int. J. Cancer* 26, 723-731 (1980).
30. Quinones, R. R., Lunney, J. & Gress, R. E. *Transplantation* (in the press).

Aggregation of chromaffin granules by calpactin at micromolar levels of calcium

Debra S. Drust & Carl E. Creutz

Department of Pharmacology, University of Virginia, Charlottesville, Virginia 22908, USA

Several cytosolic proteins bind to secretory granule membranes in a Ca^{2+}-dependent manner and thus may be involved in the mediation of membrane interactions during exocytosis[1,2]. One of these proteins[3], calpactin, is a tetramer consisting of two heavy chains of relative molecular mass (M_r) 36K (p36) and two light chains of 10K (p10)[4,5]. We report here that calpactin promotes the Ca^{2+}-dependent aggregation and fatty acid-dependent fusion of chromaffin granule membranes at a level of Ca^{2+} that is lower than that reported for other granule-aggregating proteins, and which parallels the Ca^{2+} requirement for secretion from permeabilized chromaffin cells[6,7]. We found subunits of calpactin to be inactive in promoting granule aggregation. Two distinct 33K proteolytic fragments of p36, differing at their N termini, also promote granule aggregation but with different Ca^{2+} sensitivities from calpactin. These differences suggest that the N-terminal portion of p36 modulates the Ca^{2+}/lipid binding sites in the core portion of p36 (ref. 5).

The heavy chain of calpactin, p36, was originally isolated as a major substrate for tyrosine kinases *in vivo* and *in vitro*[8-10], and it can also be phosphorylated by protein kinase C[11,12]. The main sites of phosphorylation for these kinases are at the N terminus of p36 (Tyr 23 and Ser 25)[11,13]. Little was known about the possible function of p36 until it was discovered that p36 is a subunit of a prominent Ca^{2+}-dependent membrane-binding protein (protein I) present in intestinal epithelium[4]. In addition to binding to membranes, the $p36_2$-$p10_2$ complex binds to actin in the presence of Ca^{2+} (ref. 4) and was therefore called calpactin I[14]. The sequence of p36 has recently been found to be ~50% identical to lipocortin, a 37K phospholipase A_2 inhibitor isolated from placenta[15-18].

A group of 23 cytosolic proteins, called chromobindins[1], have been isolated based on their ability to bind to secretory vesicle (chromaffin granule) membranes of the adrenal medulla in a Ca^{2+}-dependent way[1,2], and represent candidates for mediators of membrane interactions in exocytosis. By peptide mapping and immunological cross-reactivity, chromobindin No. 8 was identified as p36 (ref. 3). This is one of a subgroup of chromobin-