A Novel LFA-1 Activation Epitope Maps to the I Domain

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Abstract. A panel of 21 α-subunit (CD11a) and 10 β-subunit (CD18) anti-LFA-1 mAbs was screened for ability to activate LFA-1. A single anti-CD11a mAb, MEM-83, was identified which was able to directly induce the binding of T cells to purified ICAM-1 immobilized on plastic. This ICAM-1 binding could be achieved by monovalent Fab fragments of mAb MEM-83 at concentrations equivalent to whole antibody, was associated with appearance of the "activation reporter" epitope detected by mAb 24, and was completely inhibited by anti-ICAM-1 and LFA-1 blocking mAbs. The epitope recognized by mAb MEM-83 was distinct from that recognized by mAb NKI-L16, an anti-CD11a mAb previously reported to induce LFA-1 activation, in that it was constitutively present on freshly isolated peripheral blood mononuclear cells and was not divalent cation dependent for expression. The ICAM-1 binding activity induced by mAb MEM-83 was, however, dependent on the presence of Mg^{2+} divalent cations. Using an in vitro-translated CD11a cDNA deletion series, we have mapped the MEM-83 activation epitope to the "I" domain of the LFA-1 α subunit. These studies have therefore identified a novel LFA-1 activation epitope mapping to the I domain of LFA-1, thereby implicating this domain in the regulation of LFA-1 binding to ICAM-1.

LYMPHOCYTE function-associated antigen-1 (LFA-1)[1] is an α/β heterodimeric molecule (CD11a/CD18) which belongs to the $\beta 2$ leukocyte integrin family of adhesion receptors (for review see Springer, 1990; Pardi et al., 1992). LFA-1 is expressed on all leukocytes and mediates both heterotypic and homotypic cellular adhesion events between leukocytes and a broad range of other cell types that express at least one of the three counter-structures for LFA-1 thus far identified, namely, intercellular adhesion molecule-1 (ICAM-1) (Rothlein and Springer, 1986), ICAM-2 (Staunton et al., 1989), and ICAM-3 (de Fougerolles and Springer, 1992). The integrins are not constitutively avid for their ligands but exhibit a rapid and inducible change in avidity upon activation of the cells on which they are expressed. For example, in the case of LFA-1 expressed on T cells, stimulation via the T cell receptor/CD3 complex results in a transient increase in avidity of LFA-1 for ICAM-1, peaking at 10–30 min, followed by a return to low avidity by 1–2 h (Dustin and Springer, 1989; van Kooyk et al., 1989).

The mechanism by which remote triggering can generate such an "inside-out" signal and transduce a change in ligand-binding ability to the extracellular domains of LFA-1 is poorly understood. However, the cytoplasmic tails of the α and β chains of LFA-1 and other integrins have been shown to be involved in transmitting the "inside-out" signal to the extracellular domains (Hibbs et al., 1991; O'Toole et al., 1991). The ligand binding pocket of the integrins is thought to be formed by both the α and β subunits, since specific sequences contained in each subunit are necessary for binding of fibrinogen to the integrin GPIIb-IIIa, the most extensively studied integrin receptor/ligand pair (D'Souza et al., 1988, 1991; Charo et al., 1991). Ligand binding is regulated by the presence of divalent cations (Gailit and Ruoslahti, 1988; Dransfield et al., 1992*b*), which bind to the metal binding domains common to all integrins (Smith and Cheresh, 1991; Gulino et al., 1992). In contrast, no information is available on the functional role of the inserted (or "I") domain, which is present only in the α chains of the three leukocyte integrins, LFA-1, CR3/Mac-1, and p150/95 (Larson et al., 1989) and two members of the $\beta 1$ family of integrins, VLA-1 and VLA-2 (Ignatius et al., 1990; Takada and Hemler, 1989).

The integrin "I" domains belong to the superfamily of type A domain proteins (Colombatti and Bonaldo, 1991). They share homology with the prototype molecule of this superfamily, the A domain of von Willebrand factor, and several globular domains found in complement factors B and C2, as well as the extracellular matrix proteins collagen type VI and cartilage matrix protein. In von Willebrand factor multiple ligand binding sites have been mapped to the A domain repeats, including sites for platelet glycoprotein Ib, heparin, and collagen types I and III (Sadler, 1991). Although the $\beta 1$ integrins VLA-1 and VLA-2 act as collagen-binding receptors on various cell types, including melanocytes, fibroblasts, and platelets (for review see Hemler, 1990), the I domain appears not to confer similar collagen binding ability to the $\beta 2$ integrins on leukocytes. Collagen binding by T cells and monocytes was found to be associated exclusively with VLA-2, despite the fact that T cells expressed the I domain contain-

1. *Abbreviations used in this paper*: ICAM-1, intercellular adhesion molecule-1; LFA-1, lymphocyte function-associated antigen-1; PdBu, phorbol dibutyrate.

ing integrins VLA-1, VLA-2, and LFA-1, and monocytes expressed all five I domain containing integrins (Goldman et al., 1992). To date, no functional role has been recognized for the I domain in β2 integrins.

mAbs have been useful for demonstrating the existence of distinct conformational states in integrins during their conversion to the ligand-binding conformation. For example, mAbs 24 and 7E3 are antibodies that detect neo-epitopes expressed only by the high avidity conformers of the β2 integrins and CR3/Mac-1, respectively, thereby acting as integrin "activation reporters" (Dransfield and Hogg, 1989; Dransfield et al., 1992*b*; Altieri and Edgington, 1988). A second group of mAbs, represented by NKI-L16, specific for the LFA-1 α subunit (Keizer et al., 1988), and KIM-127, specific for the common β2 subunit (Robinson et al., 1992), are capable of directly inducing the ligand-binding conformation of LFA-1, in the putative absence of remote triggering and "inside-out" signals.

In this report, we screened a panel of 31 anti–LFA-1 mAbs and identified an α-subunit mAb, MEM-83, that was capable of directly inducing the ICAM-1 ligand-binding conformation of LFA-1. The epitope recognized by MEM-83 was mapped to the I domain, thereby implicating this domain in the regulation of LFA-1 ligand-binding affinity.

Material and Methods

Monoclonal Antibodies

mAb MEM-83 was generously provided by Dr. V. Horejsi and has been previously shown to recognize the α subunit of LFA-1 (Bazil et al., 1990; Schmidt, 1989; data not shown). Anti-CD11a mAbs MEM-83, 38, and 24, anti-CD8 mAb 14, anti-ICAM-1 mAb 15.2 (Dransfield et al., 1992*a*), and nonreactive control mAb 52U were purified by protein A chromatography as previously described (Dransfield and Hogg, 1989) and were used at 5–10 μg/ml. The remaining mAbs were obtained from Dr. Reinhold Schmidt from the Leukocyte Typing Workshop IV (Schmidt, 1989) and were used as ascitic fluids, diluted between 1:100 and 1:1,000. These antibodies included the anti-CD11a mAbs MHM24, 122.2A5, BU-49, GRT22, MEM-30, 2F12, F110.22, GRS3, HI111, M10, 0501, MEM-95, 25.3.1, 459, BU-17, YTH 81.5, 1524, TMD3-1, and CC5 1D7; and the anti-CD18 mAbs H-52, MHM23, TS1/18, M232, IC11, CLB-54, YFC 51.1, YFC 118.3, GRF1, and IB-4. Direct FITC conjugates of mAb 24 were prepared as previously described (Harlow and Lane, 1988) and were used at 5 μg/ml. Fab fragments were prepared by papain digestion (1% wt/wt) of intact MEM-83 (IgG1) or 38 (IgG2a), in 0.1 M sodium acetate, pH 5.5, 1 mM EDTA, 20 mM L-cysteine for 4 h at 37°C, followed by blocking with 75 mM iodoacetamide (Harlow and Lane, 1988). Any intact antibody and Fc fragments were separated from Fab fragments by extensive adsorption with protein A-coupled Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden) followed by size exclusion chromatography over a 100 × 1.9-cm Sephacryl S200 column (Pharmacia LKB Biotechnology). Purity of Fab fragments was confirmed by silver staining of SDS-PAGE gels (Silver Stain Plus, Bio Rad Labs., Hercules, CA).

Cells and Cell Lines

Activated T cells were expanded from unstimulated peripheral blood mononuclear cells by treatment with 1 μg/ml phytohaemagglutinin for 4 d, followed by culture in RPMI 1640 supplemented with 10% FCS and 20 ng/ml rIL-2, as previously described (Dransfield et al., 1992*b*). T cells were used after 1–2 wk of culture, after the removal of IL-2 from the culture medium for 2–3 d in order to induce quiescence. Such cells served as a model for the analysis of T cell activation (Cantrell and Smith, 1984). The human lymphoblastoid JY B cell and Jurkat T cell lines were maintained in RPMI 1640 supplemented with 10% FCS.

ICAM-1 Binding Assay

An ICAM-1 fusion protein consisting of the first three domains of ICAM-1 fused to a human IgG1 Fc fragment (ICAM-1Fc) was obtained from Drs. A. Berendt and A. Craig (Berendt et al., 1992) and purified by protein A chromatography. 40 μl of ICAM-1Fc (20 μg/ml) was added to each well of a flat-bottomed 96-well plate (Immulon 1, Dynatech Labs. Inc., Chantilly, VA) and centrifuged at 75 *g* for 1 min before overnight incubation at 4°C. Before the binding assay, nonspecific sites were blocked with 2.5% BSA in PBS without divalent cations (PBS-A) for 2 h at room temperature and washed four times with PBS-A and once in Hepes buffer (20 mM Hepes, 140 mM NaCl, 2 mg/ml D-glucose, pH 7.4). Quantitation of T cell binding to ICAM-1Fc plates was performed as previously described (Dransfield et al., 1992*a*). Briefly, 5×10^7 cultured T cells were labeled with 200 μCi $^{51}CrO_4^{2-}$ (Amersham Life Sciences, Amersham, U.K.) and washed three times in the appropriate assay medium supplemented with 0.2% BSA (fraction V, Sigma Chemical Company Ltd., Dorset, U.K.). The medium used in the binding assay was either RPMI 1640 or a defined divalent cation-containing medium consisting of Hepes buffer supplemented with various combinations of 1 mM each of $CaCl_2$, 1 mM $MgCl_2$, 1 mM EDTA, or 1 mM EGTA. ^{51}Cr-labeled T cells (2.5×10^5 in a 50-μl vol) were added to each well of an ICAM-1Fc–coated 96-well plate, in the presence or absence of 50 μl of mAbs MEM-83, 38, 15.2, or control mAb, incubated on ice for 15 min, centrifuged at 300 *g* for 1 min and incubated an additional 30 min at 37°C. T cells that remained bound to ICAM-1Fc plates after five washes with prewarmed RPMI were lysed in 1% Triton X-100 and the incorporated radioactivity was measured using a Betaplate counter (LKB Instruments Inc., Bromma, Sweden).

Homotypic Aggregation

2.5×10^5 cultured T cells were added in a 100-μl vol to each well of a 96-well plate and homotypic aggregation was semiquantitatively determined after 2 h at 37°C as previously described by the scoring method of Kansas and Tedder (1991). Aggregation of cells was read as follows: 0 = no cluster formation (<10% of cells aggregated); 1 = occasional clusters containing 10–20 cells (<20% of cells aggregated); 2 = 50% of cells in medium-sized aggregates with the remainder as single cells; 3 = majority of cells in medium-to-large–sized aggregates (>80% of cells aggregated); 4 = >90% of cells in large aggregates.

Immunofluorescence

Flow cytometry was performed as previously described (Dransfield et al., 1992*b*). Briefly, 10^5 cultured T cells or human cell lines, JY and Jurkat, were incubated for 20 min on ice with purified mAbs MEM-83, 38, or nonreactive control 52U (5 μg/ml), followed by a second incubation with FITC-conjugated goat anti-mouse immunoglobulin secondary antibody (50 μl of 1:100 dilution; Nordic Immunology, Netherlands). After appropriate washes, cells were then fixed in 0.2 ml 5.5% formaldehyde in PBS-A. mAb 24 was used as a 1:50 dilution of FITC-conjugated mAb (5 μg/ml) with incubation performed for 30 min at 37°C. Flow cytometric analysis of antibody-labeled cells was performed by determining mean fluorescence intensity using a FACScan (Becton Dickinson Immunocytometry Sys., Mountain View, CA) fitted with a logarithmic amplifier.

PCR Amplication of Fragments from Cloned cDNA and from cDNA Libraries

To construct the CD11a deletion series, fragments of LFA-1 cDNA were PCR amplified from a cDNA clone, 3R1, provided by Dr. T. Springer (Larson et al., 1989) and from an HBP-ALL library made in CDM8 provided by Dr. D. Simmons, Oxford. The 5′ PCR primers were designed to contain a BamHI restriction site, a Kozak sequence to maximize translational efficiency, an in-frame methionine, and a 24-bp hybridizing sequence corresponding to the 5′ end of the desired fragment (Stanley, P., J. E. Harvey, P. A. Bates, R. I. Bennet, and N. Hogg, manuscript in preparation). The 3′ primer consisted of the hybridizing sequence plus a HindIII restriction site. In this manner, a "nested" series of constructs with successive domain deletions starting from the NH_2 terminus of the LFA-1 α subunit was generated with each fragment terminating within domain VII (see Fig. 8 *A*). PCR amplifications were carried out using a GeneAmp™ DNA amplification kit with Taq DNA polymerase (Perkin-Elmer Corp., Norwalk, CT) according to the manufacturer's instructions. Thirty amplification cycles consisting of 2 min at 94°C, 2 min at 59°C, and 9.9 min at 70°C were used, followed by a 9.9-min extension at 70°C.

In vitro Transcription–Translation

Transcription and translation of the nested CD1 la cDNA deletion series were

carried out using the one-step TNT™ T7-coupled reticulocyte lysate system (Promega Corp., Madison, WI) according to manufacturer's instructions. 1 μg of cloned DNA in Bluescript™ KS+ phagemid served as the template for the reaction, which was carried out for 2 h at 30°C, in a 50-μl vol and in the presence of [^{35}S]methionine (Amersham Corp.) and 50% by volume rabbit reticulocyte lysate.

Immunoprecipitation of Translation Products

After translation, 25 μl of 2× EIA buffer (500 mM NaCl, 100 mM Hepes, pH 7.0, 0.2% NP-40) was added to an equal volume of translation product and the total volume of the precipitation made up to 200 μl with 1× EIA buffer. The primary incubation was carried out in the presence of 3 μg of purified mAbs MEM-83 or 14 at 4°C for 3–5 h, before the addition of 20 μl (packed volume) of Sepharose-protein A beads. The immunoprecipitations were incubated an additional 3–5 h at 4°C before harvesting the Sepharose beads by a brief 30-s microcentrifugation. 10 μl of the supernate was removed for monitoring of the translation efficiency of each fragment by SDS-PAGE. The remaining lysate was discarded and the beads washed three times with 1× EIA buffer plus 0.1% NP-40, and twice with 1× EIA buffer alone. Approximately 25 μl of electrophoresis reducing buffer was added to the beads before SDS-PAGE analysis on a 9% acrylamide separating gel. Gels were treated with EN^{3}HANCE™ (New England Nuclear, Boston, MA) before autoradiography.

Results

The Anti-CD11a mAb MEM-83 Directly Induces T Cell Binding to ICAM-1

Homotypic aggregation induced by mAbs and by phorbol esters has been previously used as a model of LFA-1/ICAM-1–dependent cell adhesion (Patarroyo et al., 1985; Rothlein and Springer, 1986; Keizer et al., 1988; Kansas and Tedder, 1991; Koopman et al., 1990). In this study, a panel of 21 α-subunit (CD11a) and 10 β-(CD18) subunit anti–LFA-1 mAbs were screened for their ability to induce homotypic aggregation and binding of T cells to ICAM-1 immobilized on plastic. None of the β-subunit mAbs in the panel induced either aggregation or ICAM-1 binding (Fig. 1). Although 12 of the 21 α-subunit mAbs were capable of inducing homotypic aggregation, only one of these, mAb MEM-83, induced ICAM-1 binding. Therefore, homotypic aggregation induced by anti-CD11a mAbs was not predictive of ICAM-1 binding.

ICAM-1 Binding Induced by mAb MEM-83 Is Specific

To further investigate the means by which bivalent antibodies caused homotypic aggregation and ICAM-1 binding, monovalent Fab fragments were made from mAbs MEM-83 and 38. mAb 38 was chosen as a representative anti-CD11a mAb that could induce homotypic aggregation of T cells but not binding to ICAM-1. Other aggregating mAbs were not available in sufficient quantities to carry out similar analysis. In light of the recent findings by Cyster and Williams (1992) that Fab fragments of anti-CD43 mAbs could spontaneously aggregate in solution and mediate a multivalent homotypic aggregation event in lymphocytes, we took great care to use Fab fragments immediately after chromatographic purification on a Sephacryl S200 size exclusion column. Fab fragments eluted from the column at a molecular mass of 50 kD and were free of any intact antibody or multivalent aggregates by silver staining of SDS-PAGE gels under nonreducing conditions (Fig. 2). Fab fragments of mAb MEM-83 were capable of inducing both homotypic aggregation and ICAM-1 binding, both effects being completely inhibited by anti–ICAM-1 mAb 15.2 (Fig. 3). Binding of T cells to ICAM-1 was also blocked with selected anti–LFA-1 mAbs (data not shown). Titration curves demonstrated that Fab fragments of mAb MEM-83 were equivalent to whole antibody in their ability to bind T cells by FACS® analysis (data not shown) or to promote T cell binding to ICAM-1 on plastic, at concentrations as low as 50 ng/ml (Fig. 4). The kinetics of the ICAM-1 binding response induced by mAb MEM-83 were similar to those observed for phorbol esters, consisting of maximal binding by 10–30 min of treatment followed by a plateau (data not shown). In contrast to the results obtained with mAb MEM-83, Fab fragments of mAb

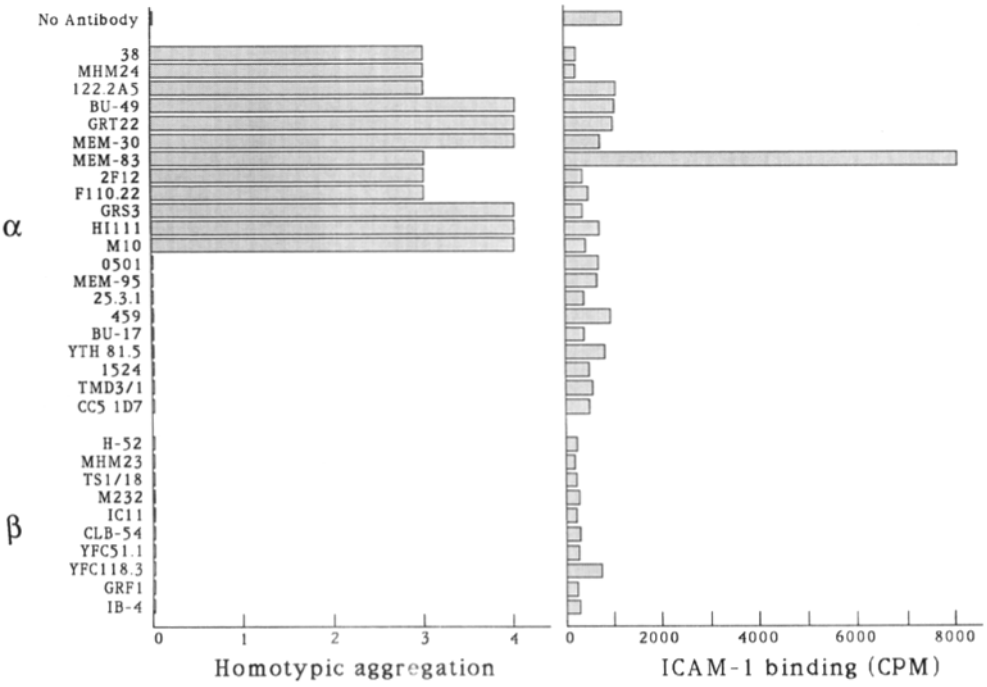

Figure 1. Screen of CD11a and CD18 panel of mAbs for homotypic aggregation and ICAM-1 binding by T cells. Human T cells were treated with saturating doses of the indicated mAbs and evaluated after 2 h of incubation at 37°C for homotypic aggregation (scale of semi-quantitative scoring = 0–4, as described in Materials and Methods) and after 30 min for binding of ^{51}Cr-labeled cells to ICAM-1Fc–coated 96-well plates (quantitated as cpm of the bound T cell fraction). Representative results from a single experiment are shown (minimum $n = 2$ for any mAb) with each bar representing the average of incubations performed in triplicate.

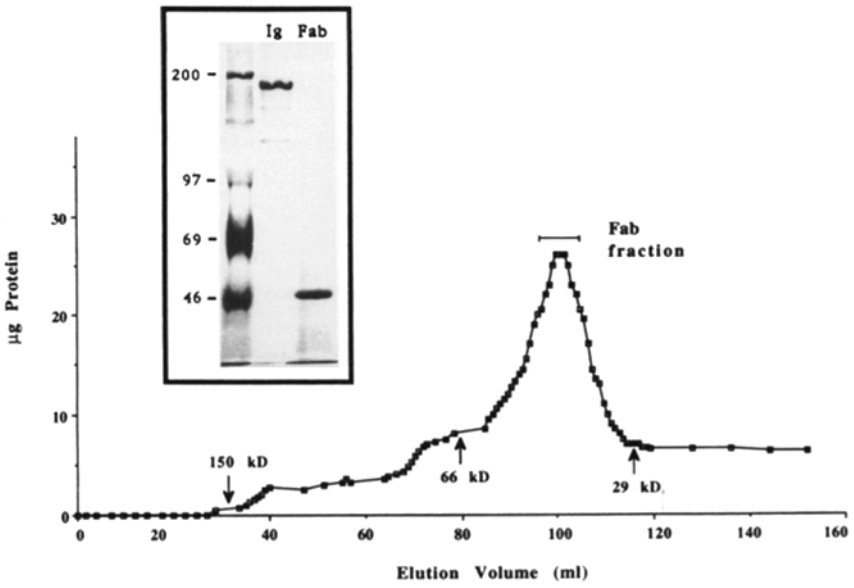

Figure 2. Purification of Fab fragments by size exclusion chromatography. Fab fragments of MEM-83 were generated by papain digestion of intact antibody, removal of Fc fragments and undigested antibody by protein A–Sepharose beads, followed by size exclusion chromatography over a Sephacryl S200 column as shown. The molecular size markers used during chromatography were alcohol dehydrogenase (150 kD), BSA (66 kD), and carbonic anhydrase (29 kD). The inset depicts 2 μg mAb MEM-83, as intact antibody (Ig) or as eluted Fab fragments (Fab), analyzed on a 7.5% SDS-PAGE gel run under nonreducing conditions and silver stained. The molecular size markers are indicated in the first lane.

38 were unable to induce homotypic aggregation and aggregates formed using whole antibody were not inhibited by anti–ICAM-1 antibody (Fig. 3). These results suggested that mAb MEM-83 was capable of directly inducing a conformation of LFA-1 that exhibited an increased avidity for ligand ICAM-1, an effect that was independent of any antibody-mediated cross-linking properties. mAb 38, on the other hand, required bivalent antibody to cause formation of homotypic aggregates and demonstrated no ability to directly induce binding to ICAM-1.

mAb MEM-83 Induces Expression of the "Activation Reporter" 24 Epitope on LFA-1

To further confirm that mAb MEM-83, but not 38, was capable of directly inducing the ligand-binding conformation of LFA-1, we examined expression of the "activation reporter" 24 epitope on LFA-1 (Dransfield et al., 1992*b*). This experiment demonstrated clearly that preincubation of T cells with MEM-83, but not 38, resulted in exposure of the 24 neoepitope on LFA-1 (Fig. 5). Nonreactive mAb 52U and phorbol dibutyrate (PdBu) treatment were included as negative and positive controls of 24 epitope expression. Cross-blocking of the 24 epitope by MEM-83 or 38 was ruled out in experiments that examined the effects of either mAb on the binding of mAb 24 in the presence of 100 μM Mn^{2+} (data not shown), which causes constitutive 24 epitope expression (Dransfield et al., 1992*b*).

Expression of the MEM-83 Epitopes on Lymphoid Cells Is Not Cation Dependent

The anti-CD11a mAb NKI-L16, which has previously been reported to possess LFA-1 activating properties, recognizes an epitope that is Ca^{2+} dependent and is weakly expressed on fresh peripheral blood T cells, though well expressed on all cultured lymphoid cells (van Kooyk et al., 1991). In contrast to NKI-L16, the MEM-83 epitope was found to be con-

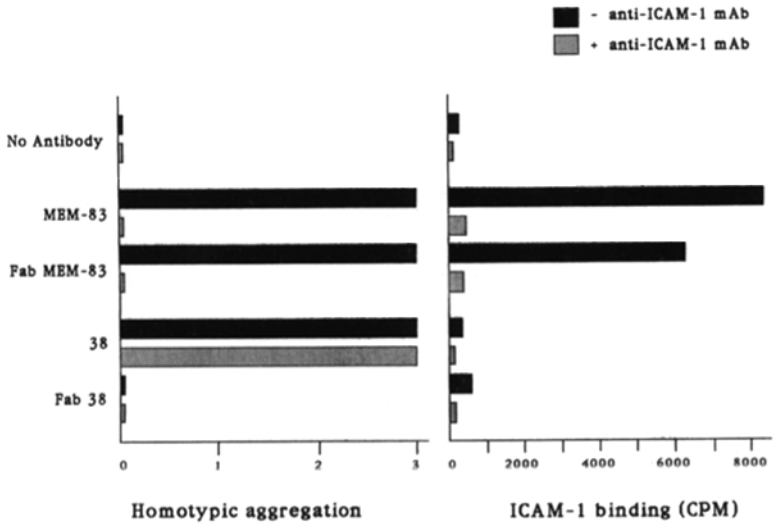

Figure 3. Effect of monovalent Fab fragments of mAbs MEM-83 and 38 on homotypic aggregation and ICAM-1 binding. The homotypic aggregation and ICAM-1 binding assays were carried out as described in Fig. 1, in the presence and absence of anti–ICAM-1 mAb 15.2 (5 μg/ml). Results from a single experiment are shown; each bar represents the average of incubations performed in triplicate. Purified intact mAb and Fab fragments were used at saturating doses of 2 μg/ml.

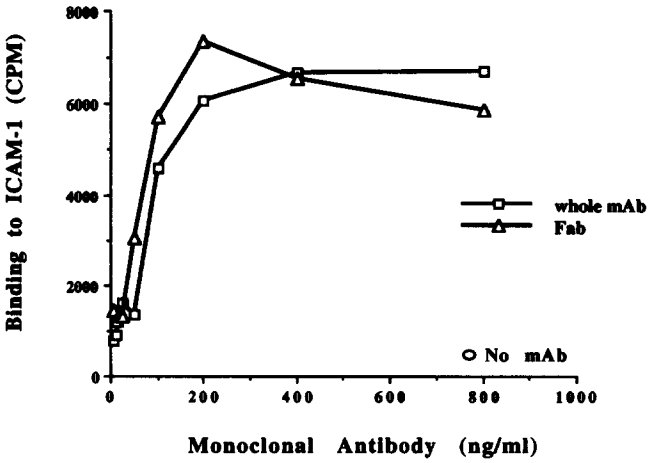

Figure 4. Titration of the ICAM-1 binding-effect induced by intact antibody and Fab fragments of mAb MEM-83. Monovalent Fab fragments of mAb MEM-83 were prepared as described in the legend to Fig. 2. Flow cytometric analysis shows that Fab fragments of MEM-83 recognize LFA-1 on T cells at titers equivalent to whole antibody (data not shown). Results from a single experiment are shown, representative of three similar experiments.

stitutively expressed on freshly isolated lymphocytes and monocytes and all human leukocytic cell lines studied, including the JY B cell and Jurkat T cell lines (data not shown).

The binding of mAb MEM-83 to cultured T cells was also examined under defined divalent cation conditions, which included the presence of both Ca^{2+} and Mg^{2+}, Ca^{2+} only, Mg^{2+} only, and the absence of both divalent cations. These experiments were carried out in the presence of two control anti-CD11a antibodies. mAb 24, which has been shown to be Mg^{2+} dependent for binding to T cells, and mAb 38, which is independent of divalent cations for its binding specificity (Dransfield and Hogg, 1989). The two control antibodies exhibited their expected cation dependency in this experiment. The results depicted in Fig. 6 showed clearly that mAb MEM-83 staining was indistinguishable from mAb 38, indicating that mAb MEM-83 was not dependent upon divalent cations for recognition of its epitope.

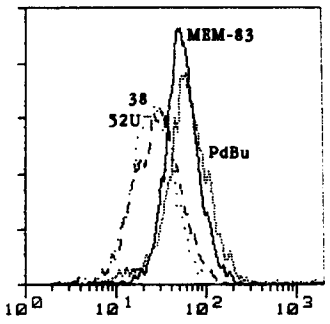

Figure 5. MEM-83 induces expression of the "activation reporter" 24 epitope on LFA-1. T cells were preincubated at 37°C for 30 min in the presence of 2 μg/ml of purified mAbs indicated, or 50 nM PdBu, in Hepes-buffered medium containing 1 mM Mg^{2+} and 1 mM EGTA. Cells were then washed and stained with FITC-conjugated mAb 24 for immunocytometric determination of 24 epitope expression. Nonreactive mAb 52U and PdBu were included as negative and positive controls of 24 expression. Immunofluorescence histograms of a single experiment are shown, representative of four similar experiments.

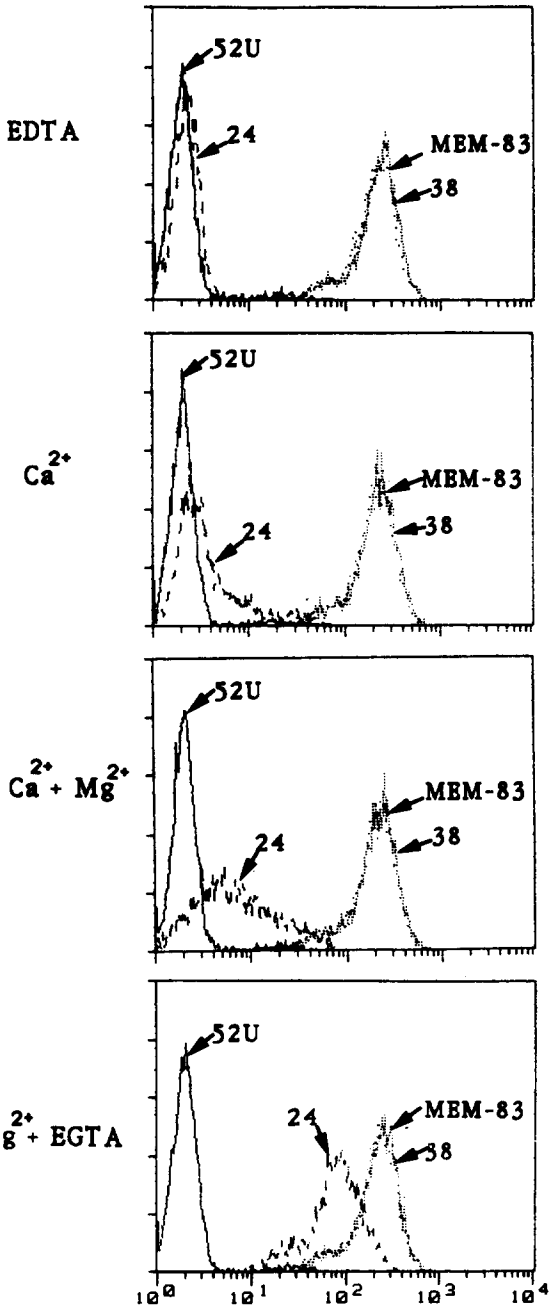

Figure 6. Expression of the MEM-83 epitope is not Ca^{2+} or Mg^{2+} dependent. T cells were preincubated at 37°C for 30 min in Hepes-buffered medium supplemented with appropriate additions of 1 mM $CaCl_2$, $MgCl_2$, EDTA, or EGTA. Divalent cation–conditioned cells were subsequently evaluated for immunofluorescence, as described in the legend to Fig. 5, using primary mAbs MEM-83, 24, 38, and 52U. Control mAbs 24 and 38 recognize epitopes that have been previously shown to be dependent and independent, respectively, of Ca^{2+} and Mg^{2+} cations (Dransfield and Hogg, 1989). Results of a single experiment are shown, representative of two similar experiments.

mAb MEM-83 Requires the Presence of Mg^{2+} to Exert Its ICAM-1 Binding Effect

The high affinity interaction between LFA-1 on T cells and ICAM-1 has previously been shown to be regulated by the presence of divalent cations in the extracellular medium,

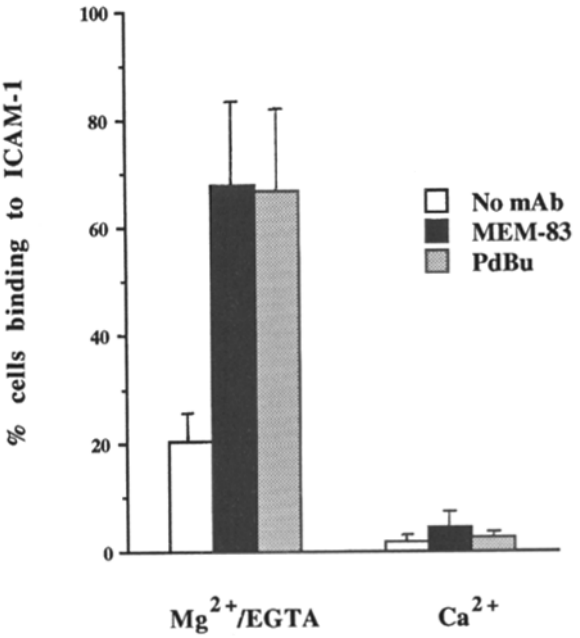

Figure 7. MEM-83–induced binding of ICAM-1 requires Mg^{2+}. T cells were labeled with ^{51}Cr and conditioned in medium containing 1 mM $CaCl_2$ or $MgCl_2$/EGTA, as described in the legend to Fig. 6. Cells were subsequently evaluated for their ability to bind ICAM-1Fc–coated 96-well plates at 37°C for 30 min, in the absence of stimuli or in the presence of 2 μg/ml mAb MEM-83 or 50 nM PdBu. Bars represent the average ± SD percentage of T cells binding to ICAM-1 ($n = 4$), relative to total counts obtained by detergent lysis.

particularly Mg^{2+} (Marlin and Springer, 1987; Dransfield et al., 1992*a*). We therefore investigated whether mAb MEM-83 could induce T cells to bind ICAM-1 in the presence of either of these divalent cations. These experiments demonstrated clearly that, despite exhibiting no divalent cation dependency for recognition of its epitope, mAb MEM-83 did require the presence of Mg^{2+} to induce binding to ICAM-1 (Fig. 7), a requirement that it shares with other known stimulators of the LFA-1/ICAM-1 adhesion pathway, such as PdBu (Martz et al., 1980; Rothlein and Springer, 1986).

mAb MEM-83 Maps to the I Domain

To identify sequences within the extracellular domain of the LFA-1 α subunit that might be involved in regulating the avidity of ligand binding, we mapped the MEM-83 epitope using an in vitro–translated CD11a cDNA deletion series. The cDNA deletion series used in these mapping studies was designed to create a series of protein fragments containing successive single-domain deletions, beginning from the NH_2 terminus of the LFA-1 α chain (Fig. 8 *a*). Protein fragments were radiolabeled with [^{35}S]methionine during in vitro translation, immunoprecipitated with mAb MEM-83 or an irrelevant anti-CD8 mAb 14, and immunoprecipitated proteins separated by SDS-PAGE. This mapping approach has been previously used to localize the epitopes of several functionally distinct anti-CD11a mAbs (Stanley, P., J. E. Harvey, P. A. Bates, R. I. Bennet, and N. Hogg, manuscript in preparation). The results depicted in the upper panel of the autoradiogram in Fig. 8 *b* demonstrated that mAb MEM-83 immunoprecipitated protein fragments containing the I domain, but not fragments from which the I domain was deleted. Nonspecific binding with mAb 14 was minimal. Efficiency of translation and incorporation of radiolabel by each protein fragment was checked by analysis of the nonimmunoprecipitated fraction of the translation mixes and was shown to be comparable across the entire deletion series (Fig. 8 *B, lower panel*). Although in vitro translation generated some truncated protein fragments in addition to the full-length translation products, only full-length products (marked by an arrow) were immunoprecipitated by mAb MEM-83. To show that the I domain is both necessary and sufficient for recognition by mAb MEM-83, a 3′ deletion fragment containing only domain II plus the I domain was constructed and also shown to be immunoprecipitated by mAb MEM-83 (data not shown). The LFA-1 activation epitope recognized by mAb MEM-83 therefore mapped to the I domain.

Discussion

From a panel of 21 CD11a and 10 CD18 mAbs, we have identified a unique anti-CD11a mAb, MEM-83, which directly induces LFA-1 binding to ICAM-1. This ICAM-1 binding could also be achieved by monovalent Fab fragments of mAb MEM-83 at concentrations equivalent to whole antibody, was associated with appearance of the 24 "activation reporter" epitope on LFA-1, and could be inhibited by anti–ICAM-1 and anti–LFA-1 blocking antibodies. Similar observations have been made for the platelet integrin gpIIb-IIIa, in which case the two mAbs P41 and Ab62 are able to induce binding to ligand fibrinogen and expression of an activation epitope PAC-1 (Shattil et al., 1985; O'Toole et al., 1990).

In contrast to the results obtained using purified ICAM-1 as a screen for LFA-1/ICAM-1–dependent adhesion, it was notable that the parallel screen using the assay of mAb-induced homotypic aggregation generated a large number of "false positive" activating mAbs. In this model, 11 of the 12 anti-CD11a mAbs that caused aggregation were not able to induce binding to immobilized ICAM-1. In the case of one such false positive, mAb 38, we were able to demonstrate that homotypic aggregation was caused by cross-linking events associated with bivalent antibody; inasmuch as homotypic aggregation could not be achieved by monovalent Fab fragments of 38, aggregation with whole antibody was not accompanied by expression of the "activation reporter" 24 epitope and aggregation was not inhibited by anti–ICAM-1 blocking antibodies. It remains theoretically possible that some of the false positive mAbs induced a conformation of LFA-1 which conferred binding exclusive to counter-receptors other than ICAM-1, such as ICAM-2 and ICAM-3. This possibility remains to be tested with appropriate blocking antibodies. Alternatively, the conclusion that bivalent mAb 38 mediates homotypic aggregation in a cross-linking–dependent manner is supported by the careful study of Cyster and Williams (1992), which showed an absolute requirement for mAb cross-linking in homotypic lymphocyte aggregation triggered by anti-CD43 mAbs. Moreover, several recent studies have invoked a role for unknown adhesion structures to explain homotypic aggregation events triggered by bivalent antibodies without rigorously excluding the possibility that these events could be mediated by cross-linking epitopes on adja-

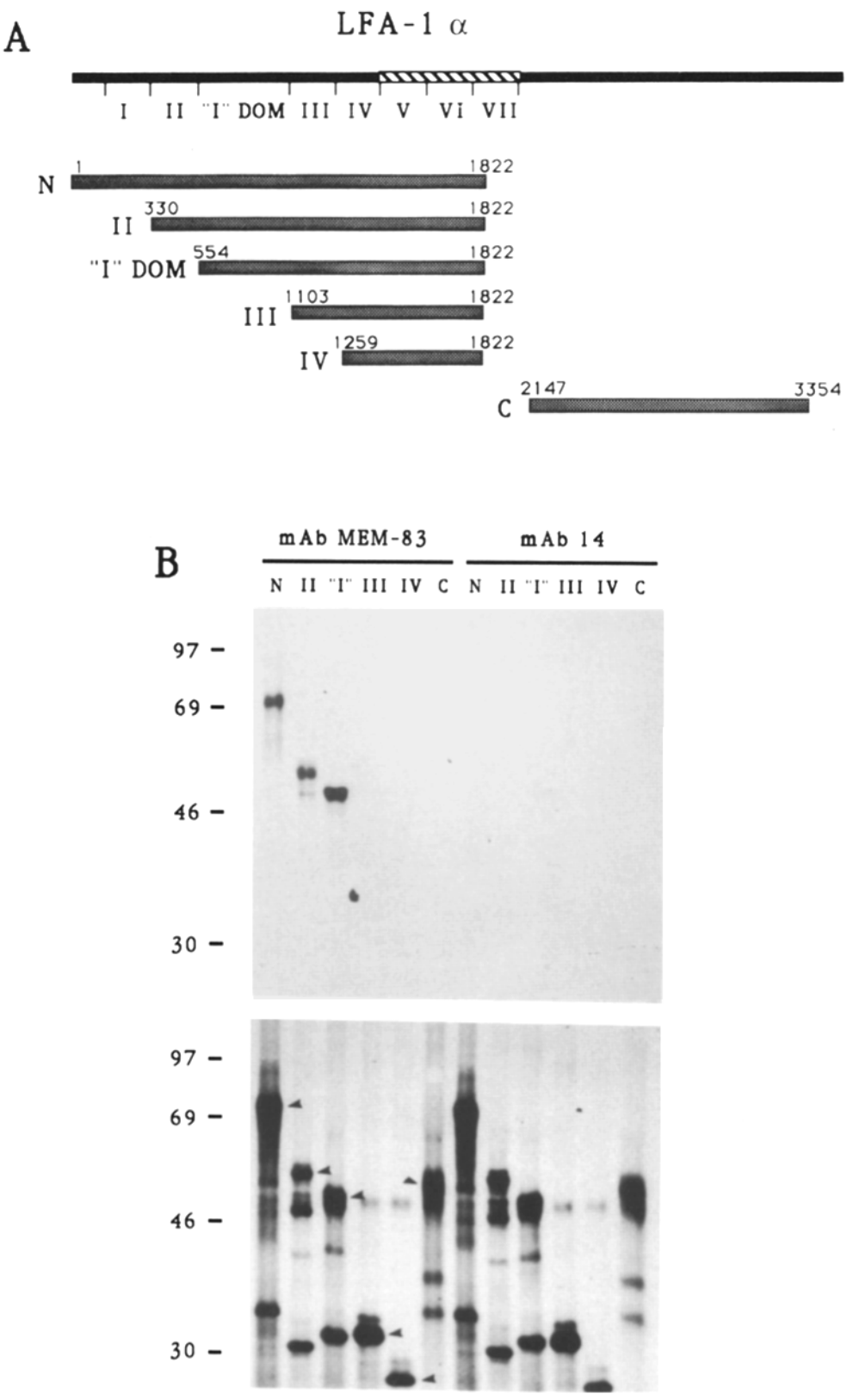

Figure 8. The MEM-83 epitope maps to the I domain of the LFA-1 α subunit. (*A*) The cDNA deletion series used in these mapping studies consisted of five NH_2-terminal fragments and one COOH-terminal fragment of the LFA-1 α subunit. The NH_2-terminal series (labeled *N, II, "I" DOM, III,* and *IV*) was constructed to contain successive single domain deletions, beginning from the NH_2 terminus of the LFA-1 α subunit and each fragment terminating in domain VII. The COOH-terminal fragment (*C*) spanned from the end of the repeating domains to the transmembrane region. Numbers designate the nucleotide position of the 5′ and 3′ termini of each cDNA construct. Domain boundaries were assigned according to the designations of Larson et al. (1989). (*B*) The cDNAs depicted in Fig. 8 *A* were used to create a corresponding series of protein fragments by in vitro transcription and translation in the presence of [^{35}S]methionine. Radiolabeled protein fragments were immunoprecipitated by mAb MEM-83 or irrelevant anti-CD8 mAb 14, harvested on protein A-Sepharose, washed, and analyzed on a 9% SDS-PAGE gel run under reducing conditions (*upper panel*). Efficiency of translation and incorporation of radiolabel by each fragment was checked by SDS-PAGE of the nonimmunoprecipitated fraction of each translation mix (*lower panel*). Protein fragments corresponding to the expected molecular size for a full-length translation product are indicated by arrows. Autoradiograms from a single experiment are shown, representative of five similar experiments.

cent cells. This present study underlines the importance of using rigorously purified Fab fragments when using antibodies to define novel activation epitopes on adhesion molecules.

Several previous studies have shown that homotypic aggregation triggered by phorbol esters served as a good model of LFA-1/ICAM-1-dependent adhesion and could be used to screen for anti-LFA-1 mAbs (Rothlein and Springer, 1986; Cobbold et al., 1987; Schmidt, 1989). Our observations are not in conflict with these past reports, in that when we used

phorbol esters to induce homotypic aggregation of T cells, the aggregation screen correctly identified 18 anti-LFA-1 (CD11a/CD18) blocking mAbs (including all previously reported blocking mAbs) without any false positives (data not shown). These results suggest that the activation state of the cell may be a crucial factor in determining the aggregation phenotype of the mAb and LFA-1/ICAM-1 dependency of the adhesion process observed. The reason for such activation dependence in homotypic adhesion processes is not yet understood but it can be speculated to be due to differences in the distribution or clustering of adhesion receptors. Homotypic aggregation should therefore be used with caution as a model for LFA-1/ICAM-1–dependent adhesion.

A comparison of the activation epitopes recognized by the CD11a-specific mAbs MEM-83 and NKI-L16 (Keizer et al., 1988; van Kooyk et al., 1991) indicated that the two antibodies recognized functionally distinct epitopes. Whereas the NKI-L16 epitope has been reported to be present only at low levels on freshly isolated leukocytes, the MEM-83 epitope was constitutively expressed on freshly isolated T cells and monocytes. In addition, expression of the MEM-83 epitope was completely independent of the divalent cations Ca^{2+} and Mg^{2+}, whereas the NKI-L16 epitope was Ca^{2+} dependent for its recognition. Furthermore, using an in vitro–translated CD11a cDNA deletion series, we were able to map the activation epitope recognized by mAb MEM-83 to the I domain of the LFA-1 α subunit. In contrast, evidence derived from tryptic cleavage studies suggested that the NKI-L16 epitope may be located close to the membrane (Keizer et al., 1988).

Localization of the MEM-83 activation epitope to the I domain of the LFA-1 α subunit suggests for the first time that this domain may be involved in regulating the interconversion between the high- and low-avidity ligand-binding conformations of LFA-1. Although models for integrin structure are still at a preliminary stage (Calvete et al., 1992), it can be speculated that the I domain inserted between LFA-1 domains II and III might be in a position to influence the "availability" of the divalent cation binding domains V-VII, perhaps by inducing an allosteric alteration in the tertiary folding of LFA-1 and either revealing these domains or altering their ability to bind divalent cation.

A further speculation would be that binding of mAb MEM-83 may achieve from the exterior of the cell a similar alteration in the tertiary structure of LFA-1 as the "inside-out" signal triggers from within the cell. Thus, binding of mAb MEM-83 may lead to exposure, via allosteric changes to the tertiary structure, of ligand contact points anywhere on the LFA-1 receptor including the I domain itself. Indeed the three mAbs which can cause LFA-1 activation—MEM-83, NKI-L16, and KIM-127—all interact with separate epitopes on the α and β subunits, suggesting that very widespread alterations in LFA-1 conformation occur upon activation and that changes affecting ligand binding ability might take place at sites distant from the activation epitopes.

In summary, we have identified an anti-CD11a mAb, MEM-83, which is capable of directly inducing the ICAM-1 binding conformation of LFA-1. The activation epitope defined by mAb MEM-83 was mapped to the I domain of the LFA-1 α subunit, suggesting for the first time that this domain may play either a regulatory or even a direct ligand binding role in the binding of ICAM-1 by LFA-1. Studies designed to more precisely delineate the involvement of the I domain during LFA-1 activation are currently in progress.

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