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Integrin β1 Cytoplasmic Domain Dominant Negative Effects Revealed by Lysophosphatidic Acid Treatment

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Integrin receptors localize to focal contact sites and interact with the cytoskeleton via the β1 cytoplasmic domain. To study the role of this domain in adhesion, we have expressed in NIH 3T3 cells a cDNA consisting of the interleukin 2 receptor α subunit extracellular and transmembrane domains, connected to the integrin β1 cytoplasmic domain (IL2R-β1). Since the extracellular domain of the chimeric protein has no role in adhesion, this protein could uncouple adhesion from intracellular events. As expected, in a cell line expressing IL2R-β1, this chimera was directed to focal contact sites. Unexpectedly, the cells exhibited normal adhesion to fibronectin (FN). However, when a rapid reorganization of the cytoskeleton was induced using lysophosphatidic acid (LPA), IL2R-β1 cells detached from FN in contrast to wild-type cells. The detachment in response to LPA could be prevented with cytochalasin D, an inhibitor of actin polymerization. These results imply that a β1 cytoplasmic domain, which is uncoupled from adhesion, can compete with the cytoplasmic domain of native integrin β1 for cytoskeletal proteins. As a consequence, the IL2R-β1 protein acts as a dominant negative effector of adhesion by disrupting the integrin-cytoskeleton connection.

INTRODUCTION

Integrin receptors each consist of an α- and a β-subunit that associate to mediate interactions between cells and the extracellular matrix (ECM). Many integrin receptors that are involved in adhesion concentrate in specific regions within the plasma membrane after having bound their ECM substrates. These specialized domains of the plasma membrane are recognized in interference reflection microscopy as dark areas and are called focal contact sites (Burridge *et al.*, 1988). The actin microfilament network is anchored at focal contact sites, which also contain other components of the cytoskeleton, such as vinculin, α-actinin, and talin (Chen *et al.*, 1985; Damsky *et al.*, 1985; Dejana *et al.*, 1988; Singer *et al.*, 1988). The latter two proteins have been shown to interact with the cytoplasmic domain of the β1 subunit in vitro, suggesting a direct interaction in vivo (Horwitz *et al.*, 1986; Otey *et al.*, 1990). Thus integrins are thought to provide a mechanical linkage between the ECM and the cytoskeleton.

All integrin subunits contain a large extracellular domain, a single transmembrane segment and a carboxyl terminal cytoplasmic domain of varying length. The β1 cytoplasmic domain has been shown to be required for integrin focal contact localization (Solowska *et al.*, 1989; Hayashi *et al.*, 1990; Marcantonio *et al.*, 1990; Reszka *et al.*, 1992). Furthermore, it has been shown that the β1 cytoplasmic domain itself is able to localize chimeric reporter-β1 proteins to focal contacts (Geiger *et al.*, 1992; LaFlamme *et al.*, 1992). This domain is not only necessary, but appears to be sufficient to localize integrin to focal contact sites. However, in vivo, all integrin localization to focal contact sites requires ligand binding (Dejana *et al.*, 1988; Singer *et al.*, 1988). It has been shown that the α-cytoplasmic domain may play a role in the control of localization of integrins to focal contacts (Briesewitz *et al.*, 1993; Ylänne *et al.*, 1993). Deletion of most of the α1 cytoplasmic domain (preserving the conserved GFFKR sequence) leads to the localization of this

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integrin to focal contacts even on substrates which $\alpha 1\beta 1$ does not bind. Thus loss of the α cytoplasmic domain leads to ligand-independent focal contact localization. These experiments suggest that in native integrins, the α cytoplasmic domain may mask the $\beta 1$ cytoplasmic domain, preventing ligand-independent focal contact localization. Recently, we have shown that the α- and β-cytoplasmic domains are associated, and that this association is altered upon ligand binding in the normal function of integrins, allowing post-ligand binding events to proceed (Briesewitz, Kern, and Marcantonio, unpublished data).

We are interested in the mechanism of focal contact formation, and have decided to use a chimeric protein consisting of the extracellular and transmembrane domains of the interleukin 2-receptor and the cytoplasmic domain of the integrin $\beta 1$ (IL2R-$\beta 1$) as a probe for the intracellular function of integrins. This chimeric protein is "active", since it is localized to focal contact sites (LaFlamme *et al.*, 1992). Because the extracellular domain of the chimeric protein has no adhesive role, expression of IL2R-$\beta 1$ should uncouple adhesion from intracellular events, due to a competition with the cytoplasmic domain of native integrin $\beta 1$ molecules. Thus we expected this protein to behave as a dominant negative component in cell adhesion. We have produced stable NIH 3T3 cell lines which express IL2R-$\beta 1$, with unexpectedly normal adhesion to fibronectin (FN)[1]. However, the cells rounded-up upon treatment with serum supplemented media. We have characterized this inducible dominant negative effect and now show that it is also seen upon treatment with lysophosphatidic acid (LPA). In this study, we use this effect to identify the mechanism by which chimeric proteins containing the $\beta 1$ cytoplasmic domain exert their dominant negative effect.

MATERIALS AND METHODS

Plasmid Construction

Restriction enzymes and T4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA), T4 DNA ligase from Life Technologies (Gaithersburg, MD), and Taq polymerase from Boehringer-Mannheim (Indianapolis, IN). Oligonucleotides were purchased from Operon Technologies (Alameda, CA) and were used directly without further purification. The chicken integrin $\beta 1$ sequence was isolated from the cDNA clone 1D described previously (Tamkun *et al.*, 1986). The cDNA for the interleukin 2 receptor, α subunit, (Giordano *et al.*, 1991) was kindly provided by Dr. T. Giordano (Abbott Labs, Abbott Park, IL). The interleukin-$\beta 1$ cytoplasmic domain construct (LaFlamme *et al.*, 1992) was made by overlapping PCR (Horten *et al.*, 1990). The construct was subcloned in the expression vector pLEN (Marcantonio *et al.*, 1990) for constitutive expression or in pMEP for inducible expression. The sequence of the chimeric DNA was confirmed by dideoxy sequencing (Sequenase; USB, Cleveland, OH), using internal primers.

DNA Transfections

NIH 3T3 cells were maintained in DME with 10% CS. Cells (5×10^5) plated the previous day, were co-transfected with 20 μg of the interleukin-$\beta 1$ cytoplasmic domain chimera and 2 μg pSV2neo (Southern and Berg, 1982) as a calcium phosphate precipitate (as described previously) (Solowska *et al.*, 1989). After 3 days, the cells were split 1:20 into DME supplemented with 10% CS and 1 mg/ml G418 (Geneticin; Life Technologies, Gaithersburg, MD). After 10–14 days, G418 resistant clones were isolated and screened by immunoprecipitation and immunofluorescence.

Antibodies

Rabbit anti-$\beta 1$ cytoplasmic domain antibodies were prepared as described (Marcantonio and Hynes, 1988). The rabbit anti-talin serum was the kind gift of K. Burridge (University of North Carolina). Monoclonal anti-human interleukin-2 receptor antibody was purchased from Amersham (Arlington Heights, IL) and monoclonal anti-mouse vinculin was purchased from Sigma Chemical (St. Louis, MO).

Radiolabeling and Immunoprecipitation

Cells were labeled with Na ^{125}I (New England Nuclear, Boston, MA) and lactoperoxidase as a monolayer as described (Hynes, 1973). Cells (10^7) and 1–2 mCi/ml were used for each experiment. The cells were extracted with 0.5% NP-40 and immunoprecipitated as described (Marcantonio and Hynes, 1988). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970). Separation gels were 7% acrylamide with a 3% stacking gel. Samples were prepared in sample buffer (5% SDS, 100 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 6.8, 10 mM EDTA, 10% glycerol and bromophenol blue) and boiled for 3 min.

Immunofluorescence

Fibronectin (1 μg) in 70 μl of water was air-dried on coverslips after which the protein was rehydrated >30 min in 1 ml of water, followed by two washes with phosphate-buffered saline (PBS). Cells were plated on the coverslips and incubated overnight. After performing the experiments, the cells were fixed in 4% paraformaldehyde in PBS for 5 min and then permeabilized with 0.2% NP-40 in PBS for 5 min. The antibodies against talin, vinculin, and interleukin receptor were diluted 1:250 in 10% normal goat serum in PBS. The coverslips were overlaid with 35 μl of primary antibody solution and incubated at 37°C for 30 min. After washing in PBS the coverslips were incubated with 20 μl of secondary antibodies for 30 min at 37°C. The secondary antibodies were F(ab')$_2$ fragments of rhodamine-conjugated rabbit anti-mouse IgG and fluorescein-conjugated goat anti-rabbit IgG (Cappel, Durham, NC). Rhodamine-labeled phalloidin was diluted 1:500 in 10% normal goat serum in PBS and incubated with fixed and permeabilized cells for 30 min at 37°C.

Cell Adhesion Assays

Adhesion assays were done essentially as described by Aumailley *et al.* (1989). FN was diluted in distilled water and allowed to dry in microtiter wells at room temperature. After washing with PBS, remaining protein binding sites were blocked with BSA (1% in PBS). Cells were suspended in serum-free DME ($4–5 \times 10^5$/ml) and incubated in the treated wells for 45 min at 37°C. Nonadherent cells were removed by washing with PBS, and bound cells were fixed with 70% ethanol and stained with crystal violet (0.1% in distilled water). Excess stain was removed with water. Bound stain was dissolved with Triton X-100 (0.2% in distilled water) and the optical density was read at 595 nm. All experiments were done in triplicate.

To assay the effect of LPA, FN (1 μg/coverslip) was diluted in distilled water and allowed to dry on glass coverslips at room temperature. Cells were suspended in complete medium and incubated on the coverslips for 16 h. Various amounts of LPA (Sigma Chemical, St. Louis, MO), 1 mM cytochalasin D (Sigma Chemical) or fresh media containing various concentrations of calf serum were added to the cells as indicated in the figure legends. The percentage of detached cells was calculated by measuring the number of attached cells (as

[1] Abbreviations used: FN, fibronectin; LPA, lysophosphatidic acid.

described above) in each sample, and then subtracting this number from the number of attached cells in the untreated samples.

Flow Cytometry

Fluorescence flow cytometric analysis was performed on a FACSscan cytofluorometer (Becton Dickinson, Mountain View, CA). The cells were trypsinized, washed with growth media, and suspended in PBS with 0.5% CS and 0.5% NaN_3 using 5×10^5 cells in 50 ml. Staining was performed with a 1:250 dilution of monoclonal anti-interleukin receptor antibody on ice for 30 min, followed by three washes with cold PBS. Cells were then resuspended and stained with FITC-conjugated goat anti-mouse antibody (TAGO) 1:100 dilution for 30 min. After three washes, cells were analyzed.

RESULTS

Expression of β1 Cytoplasmic Domain Chimeric Protein in NIH 3T3 Cells

To study the function of the β1 cytoplasmic domain, we have connected this domain to the transmembrane and extracellular domains of the interleukin-2 receptor a subunit, creating the chimeric molecule IL2R-β1. The cDNA was transfected into NIH 3T3 cells, and stable cell lines were isolated. Figure 1A shows the results of an immunoprecipitation of extracts derived from ^{125}I surface labeled IL2R-β1 cells. Immunoprecipitation with an anti-interleukin receptor antibody or an anti-β1 cytoplasmic domain antibody shows the chimeric protein with an expected molecule weight of 55 kD. In addition, integrin heterodimers typical of NIH 3T3 cells are recovered with the cytoplasmic domain antibody.

Since we expected the IL2R-β1 protein to behave as an inhibitor of integrin function, we decided to test the adhesive capacity of this cell line. The adhesive properties of IL2R-β1 cells were compared with those of NIH 3T3 cells (Figure 1B). When plated on FN under standard conditions (see MATERIALS AND METHODS), surprisingly, there was unexpectedly no difference in the adhesive properties of the IL2R-β1 cells (clone 3) as compared with NIH 3T3 cells. Similar results have been obtained with other IL2R-β1 cell clones as well as with a clone with very high expression of the full length interleukin receptor (with the native IL-2 receptor cytoplasmic domain) (corroborative data). However, this assay primarily measures initial attachment of the cells, and it is performed under conditions which do not support maximal cytoskeletal organization, that is, in the absence of serum, and with a brief incubation time (45 min).

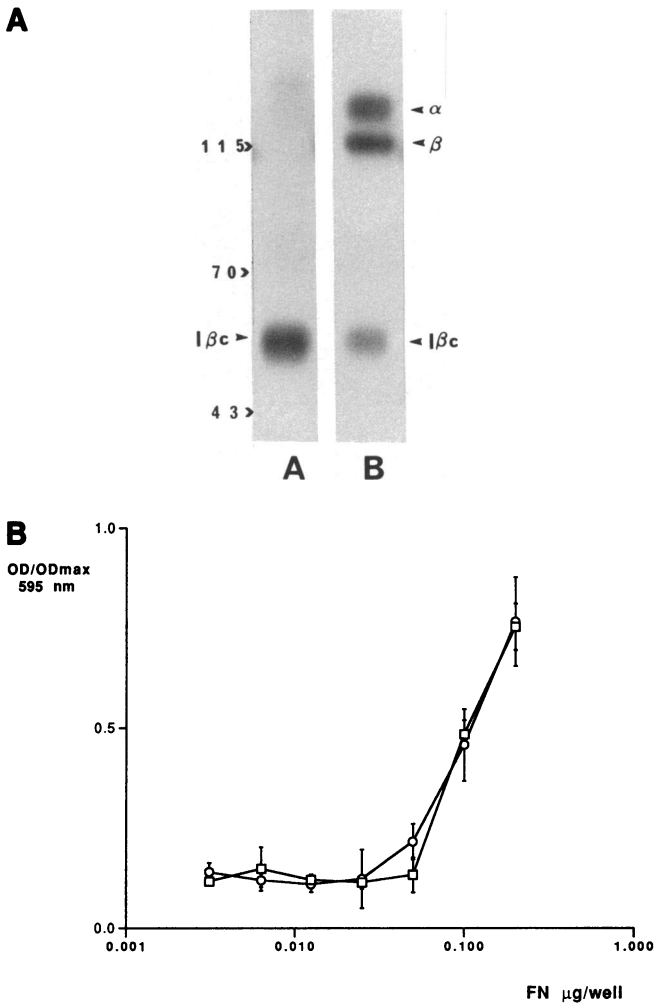

Figure 1. (A). Immunoprecipitation of surface labeled 3T3 IL2R-β1 cells. Extracts of ^{125}I surface labeled 3T3 IL2R-β1 cells were incubated with mouse monoclonal anti-interleukin receptor antibody (lane A) or rabbit anti-β_1 cytoplasmic peptide serum (lane B). Immunoprecipitates were recovered by the use of protein A-Sepharose (lane B) or by using goat anti-mouse agarose (lane A), followed by analysis using SDS-PAGE and autoradiography. (B) Adhesion assay of 3T3 cell lines. NIH 3T3 Cells (□) and IL2R-β1 #3 cells (○) were assayed for adhesion to FN. Amounts of protein indicated at the bottom of the graph were dried down on microtiter plates. Cells were incubated for 45 min at 37°C, washed, and the bound cells were fixed and stained. Optical density was read at 595 nm. Both cell lines have similar adhesion curves on FN.

Effect of Lysophosphatidic Acid (LPA) on NIH 3T3 Cells

Recently, Ridley and Hall (1992) have shown that LPA present in serum is critical for cytoskeletal organization and focal contact formation in Swiss 3T3 cells. We have investigated the effect of LPA on NIH 3T3 cells. When NIH 3T3 cells are plated on FN in the absence of serum or LPA, they have modest focal contact size and cytoskeletal organization, as shown by staining with a monoclonal anti-vinculin antibody (Figure 2a) or with phalloidin (Figure 2b). One minute after treatment with LPA, the most prominent change is an increase in the organization and number of stress fibers seen with phalloidin staining (Figure 2d). After 5 min, the size of the focal contacts appears to increase as well (Figure 2e).

Since LPA effectively promotes cytoskeletal reorganization in NIH 3T3 cells, we tested the effect of LPA treatment on IL2R-β1 cells (Figure 3). As expected,

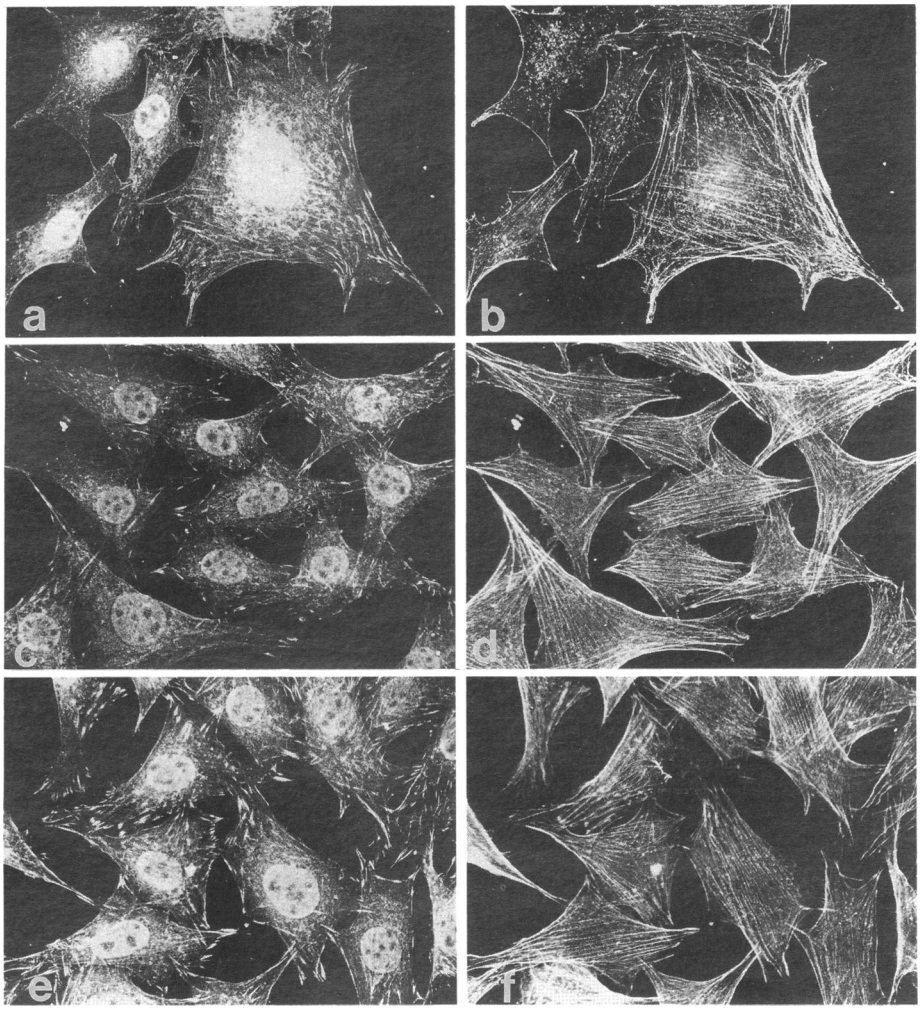

Figure 2. Effect of LPA on focal contact and stress fiber formation in NIH 3T3 cells. NIH 3T3 cells were plated on FN-coated coverslips and incubated at 37°C for 3 h. The cells were fixed before the addition of new media containing 1 μg/ml LPA (a and b), or after 1 (c and d) or 5 min (e and f) incubation time. The cells were stained with either rabbit polyclonal anti-talin serum as a focal contact marker (a, c, and e) or with rhodamine-conjugated phalloidin (b, d, and f). The primary antibody was visualized by the use of fluorescein-conjugated goat anti-rabbit IgG.

treatment of parental NIH 3T3 cells with LPA had little effect on their appearance in phase-contrast micrographs (Figure 3, a-c). In contrast, after 1 min of treatment with LPA, the IL2R-β1 containing cells began to round up on the substratum. After 2 min many of the cells had completely rounded up (Figure 3, e and f). This response to LPA is reversible, since the cells will spread again if incubated for longer times (hours), presumably due to either the metabolism of LPA, or to a down regulation of the LPA signaling pathway (corroborative data). This reversibility explains the ability to isolate stable IL2R-β1 cell lines growing in serum, which contains LPA.

This dramatic difference between the parental 3T3 cell and the IL2R-β1 cells is also seen in immunofluorescence experiments. In these experiments, a mixture of parental NIH 3T3 cells and IL2R-β1 cells was plated on FN and stained with antibodies to the focal contact protein talin, (Figure 4, a and c) as well as antibodies to the interleukin-2 receptor (Figure 4, b and d). If the cells were allowed to spread without a challenge of LPA, both populations of cells spread well on FN, and formed focal contacts (Figure 4, a and b). However, upon treatment with LPA for 1 min, the IL2R-β1 positive cells round up, while the negative cells (parental 3T3 cells) remain flat and well spread (Figure 4, c and d). This experiment demonstrates that the IL2R-β1 protein is localized in focal contacts, and that individual cells containing this chimeric protein will round up in response to LPA treatment.

Inducible Expression of the Dominant Negative Construct

One could argue that the phenotype displayed by the IL2R-β1 cells is a particular clonal variant which leads to the response to LPA. To rule out this possibility, we established 3T3 cell lines that express the IL2R-β1 cDNA under the control of the mouse metallothionein promotor, which can be induced by Zn^{2+} or Cd^{2+}. One of these lines was analyzed further. This cell line has a low basal level of expression of the chimera without added Zn^{2+}, as shown by minor focal contact staining in cells plated on FN and stained with anti-IL2 receptor

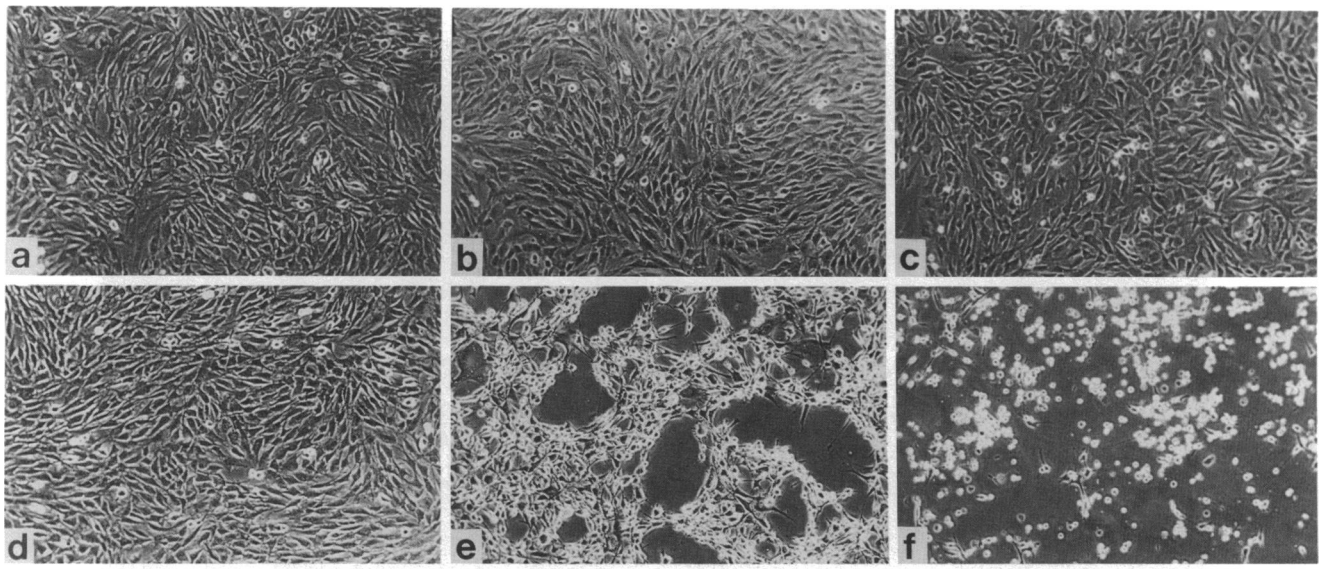

Figure 3. Effect of LPA on NIH 3T3 and 3T3 IL2R-β1 cells. NIH 3T3 cells (a-c) and 3T3 IL2R-β1 cells (d-f) were plated on FN coated coverslips and incubated in complete media at 37°C overnight. LPA (5 μg/ml) was added and cells were photographed immediately (a and d), after 2 min (b and e) or after 5 min (c and f) at room temperature. The NIH 3T3 cells remained attached throughout, while the 3T3 IL2R-β1 cells begin to detach after 2 min and detached almost completely by 5 min.

antibody (Figure 5a). Furthermore, flow cytometric analysis also shows a low basal level of expression (Figure 6B; left). Most importantly, these cells do not round up upon treatment with LPA (Figure 5c). However, upon incubation overnight in media containing 60 mM Zn^{2+}, there is induction of IL2R-β1 expression, as shown by the staining of focal contacts with anti-IL-2 receptor antibodies (Figure 5b), and by the increased fluorescent intensity in flow cytometric analysis (Figure 6B; right). Unlike the noninduced cells, the induced cells now re-

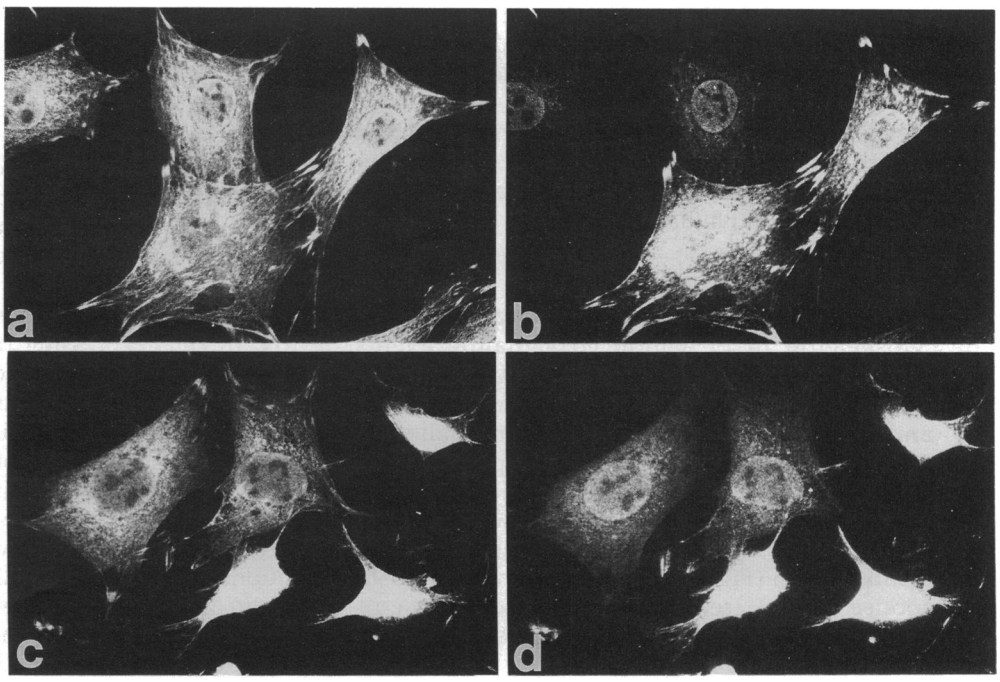

Figure 4. Immunofluorescence of a mixture of NIH 3T3 cells and 3T3 IL2R-β1 cells. Approximately equal numbers of NIH 3T3 cells and 3T3 IL2R-β1 cells were mixed and plated on FN coated coverslips in serum-free media for 3 h at 37°C. The cells were either fixed immediately (a and b) or treated with LPA (20 ng/ml) for 2 min, followed by fixation (c and d). Double-label immunofluorescence was performed using a mixture of a rabbit polyclonal anti-talin serum as a focal contact marker and the mouse monoclonal anti-interleukin receptor antibody. The primary antibodies were visualized by the use of rhodamine-conjugated rabbit anti-mouse IgG for the interleukin receptor (b and d) and fluorescein-conjugated goat anti-rabbit IgG for the anti-talin serum (a and c). The IL2R-β1 chimeric protein is localized to focal contacts on FN; these cells round-up upon LPA treatment. The IL2R-β1 negative cells remained attached with talin labeled focal contacts.

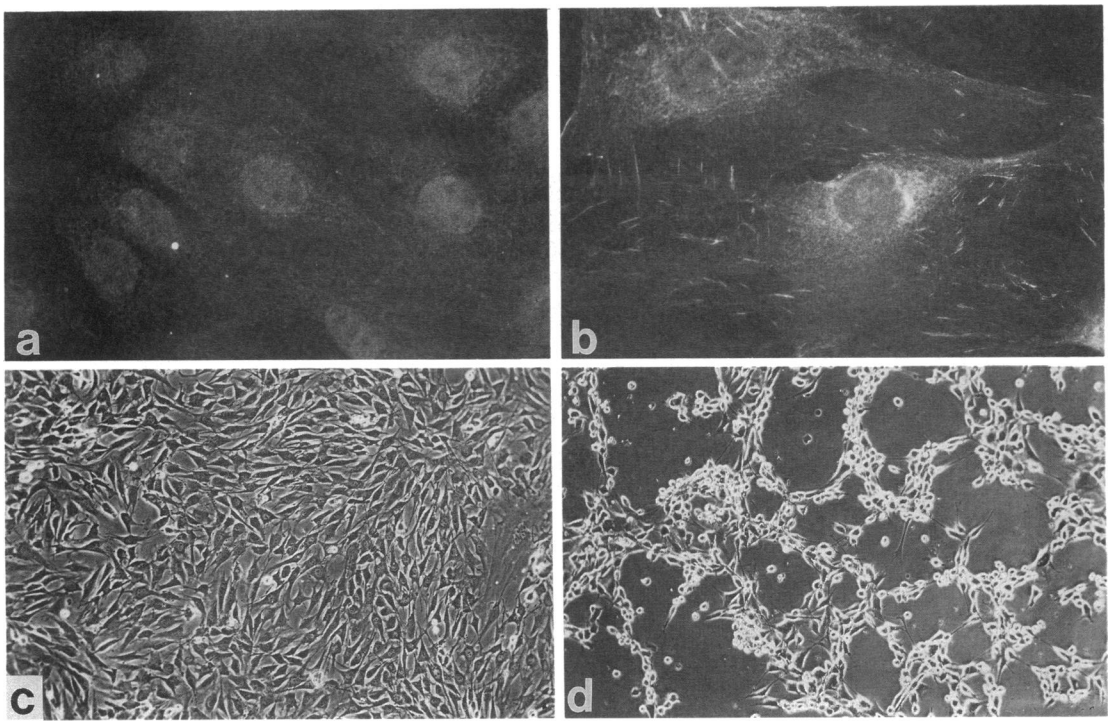

Figure 5. Induction of expression of IL2R-β1 under the control of metallothionein promotor. 3T3 cells were transfected with the vector pMEP containing the IL2R-β1 sequence under the control of the metallothionein promotor, and a cell line containing the plasmid was isolated. The cells were plated on FN coated coverslips and incubated overnight in media without added $ZnSO_4$ (a) or with 60 mM $ZnSO_4$ (b-d). Immunofluorescence was performed using the mouse monoclonal anti-interleukin receptor antibody (a and b). The primary antibody was visualized by the use of rhodamine-conjugated rabbit anti-mouse IgG. In the bottom half of the figure, 5 μg/ml LPA was added to induced cells and they were photographed either immediately (c), or after 2 min (d) at room temperature. The induced cells have IL2R-β1 in focal contacts and detach upon LPA treatment.

spond to LPA with a rapid detachment from the substratum (Figure 5d). The percentage of detached cells after LPA treatment corresponds to the amount of Zn^{2+} and the degree of induced expression of IL2R-β1 (Figure 6A). This detachment is dependent on the presence of the β1 cytoplasmic domain in the chimera, since control cell lines with very high expression of the full length interleukin receptor (with the native IL-2 receptor cytoplasmic domain) did not detach in response to LPA treatment (corroborative data).

Requirement of the Actin Cytoskeleton

To determine the role of the rapid reorganization of the cytoskeleton in LPA-induced signaling events, cells were incubated with cytochalasin D, an inhibitor of actin polymerization. IL2R-β1 cells were plated on FN, and spread well (Figure 7a). When challenged with LPA, these cells rapidly rounded-up on FN (Figure 7b). However, if IL2R-β1 cells experienced a brief treatment with cytochalasin D, they showed a slightly more elongated morphology; they remained attached to the substratum (Figure 7c), and did not round-up in response to LPA treatment (Figure 7d). These results show that the actin cytoskeleton is involved in the LPA-dependent detachment.

DISCUSSION

This study demonstrates that the cytoplasmic domain of the integrin β1 subunit can act as a dominant-negative component in cell adhesion when it is not connected to the extracellular matrix. This effect was achieved by linking the β1 cytoplasmic domain to the extracellular domain of the interleukin receptor. The resulting chimeric protein causes the detachment of NIH 3T3 cells from a fibronectin substrate when a rapid reorganization of the cytoskeleton is induced by LPA.

The β1 cytoplasmic domain presumably acts as a receptor for cytoskeletal proteins, thus establishing the link between the cytoskeleton and the extracellular matrix. In cells expressing the IL2R-β1 chimera, the additional β1 cytoplasmic domains compete for cytoskeletal proteins without contributing to adhesion. A very high expression of this chimera interferes with adhesion to such an extent that stable cell lines cannot be isolated. Consequently, the constitutive expression of IL2R-β1 in our NIH 3T3 cell lines is below the threshold that affects adhesion and growth under routine culture conditions. However, when these cells are plated on fibronectin and a rapid cytoskeletal reorganization is induced by LPA, the dominant negative effect of IL2R-β1 on cell attachment is revealed since detachment of the cells

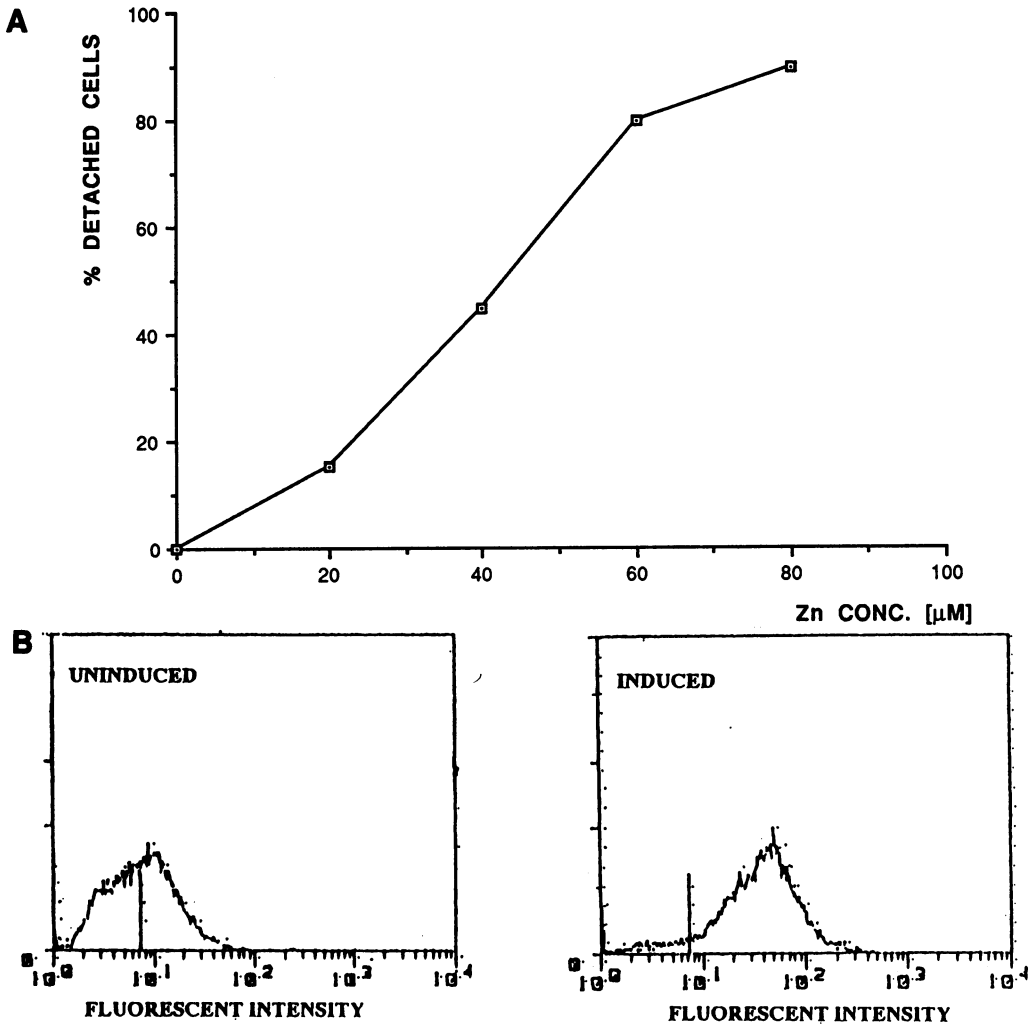

Figure 6. (A) Increasing numbers of cells detached with LPA treatment as a consequence of induction of IL2R-β1 expression. pMEP IL2R-β1 cells were plated on FN coated coverslips, and incubated overnight in media with various concentrations of $ZnSO_4$ as indicated at the bottom of the panel. These cells were then challenged with 5 μg/ml LPA and the percentage of detached cells was calculated as described in MATERIALS AND METHODS. (B) Flow cytometric analysis of uninduced and induced pMEP IL2R-β1 cells. The surface expression of IL2R-β1 was measured by flow cytometric analysis using anti-interleukin receptor antibody, before induction (left graph) and after overnight induction using 60 mM $ZnSO_4$ (right graph). There is an obvious increase in the surface expression of IL2R-β1 after induction with Zn^{2+}.

occurs. This effect is down regulated over time (hours) and the detached cells will again adhere and spread over this time period. Thus when cells are plated in the presence of serum or LPA, they will attach and spread over 3 h, and the dominant negative effect will only be revealed by the addition of fresh LPA to promote a rapid cytoskeletal reorganization. It should be noted that LPA and serum are interchangeable in these detachment experiments.

LPA is a mitogen for many cell types in culture (van Corven *et al.*, 1989). The binding to its G-protein coupled receptors leads to the hydrolysis of phosphoinositides and the release of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) causing a rapid transient rise in cytosolic Ca^{2+} and PKC activation (Jalink *et al.*, 1990; van Corven *et al.*, 1989). Cytosolic Ca^{2+} and PKC are involved in regulating microfilaments and, indeed, LPA has been shown to induce the assembly of actin stress fibers and focal contacts in Swiss 3T3 cells (Ridley and Hall, 1992). We have demonstrated a similar effect for NIH 3T3 cells. Within 1 min, a reorganization of the cytoskeleton with increased stress fiber formation was observed and after 5 min an increase of focal contact size was apparent. There was no dramatic change in the cell shape.

In contrast, when LPA was used to trigger the rapid reorganization of the cytoskeleton in IL2R-β1 expressing cells, the cells detached from the matrix. During this reorganization of the cytoskeleton, the large pool of IL2R-β1 derived β1 cytoplasmic domains withdraws critical cytoskeletal elements from integrins that are bound to the extracellular matrix. Hence, as the stress fibers expand and exert increasing tension on the cell, they are linked to IL2R-β1 and a reduced number of integrins that are not sufficient to counter the exerted force. The role of the actin cytoskeleton in causing the LPA induced detachment of IL2R-β1 expressing cells was shown via the inhibition of detachment by cytochalasin D, which prevents actin polymerization and the generation of intracellular tension. This experiment strongly suggests that the major dominant negative effect of the IL2R-β1 is due to its competition for rate limiting cytoskeletal proteins, rather than interference with integrin signaling.

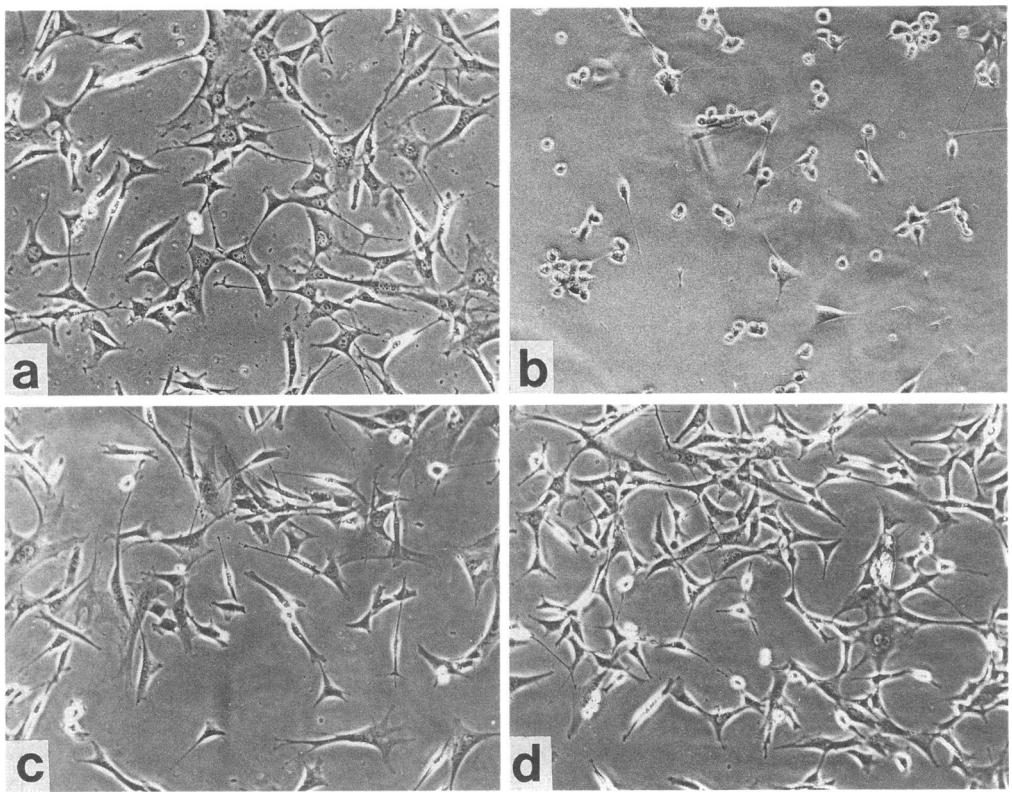

Figure 7. Effect of cytochalasin D on the LPA-dependent detachment. 3T3 IL2R-β1 cells were plated on FN coated coverslips and incubated overnight. Cells were then treated with LPA (5 μg/ml) and photographed either immediately (a), or after 2 min (b) at room temperature. In addition, cells were treated with 1 mM cytochalasin D for 2 min (c and d). Cells were either photographed immediately (c), or 2 min after treatment with LPA (5 μg/ml) (d). Strikingly, cytochalasin D pretreatment completely abolishes the LPA-dependent detachment of 3T3 IL2R-β1 cells.

Recently, it has been shown that LPA can induce the tyrosine phosphorylation of focal adhesion kinase (FAK) in quiescent fibroblasts (Kumagai *et al.*, 1993; Hordijk *et al.*, 1994). In addition, Akiyama *et al.*, (1994), have shown that IL2R-β1 constructs have the ability to stimulate FAK phosphorylation when crosslinked with anti-IL2R antibody. Thus it is conceivable that the LPA induced detachment of IL2R-β1 3T3 cells is due to signaling via these two pathways. However, in our culture system, LPA has no effect on the steady-state level of phosphorylated FAK (corroborative data). Furthermore, the cytochalasin D experiment described above clearly implicates the cytoskeletal system in the dominant negative effects we observed.

Very recently, in a similar approach, chimeric molecules with the β1 cytoplasmic domain has been used to affect cell adhesion (LaFlamme *et al.*, 1994; Lukashev *et al.*, 1994). The detachment of cells was achieved by the inducible high expression of the chimera or by transient expression. It is suggested by the authors that these domains interfere with the normal integrin-cytoskeleton association by competition for cytoskeletal elements. We show that the primary effect in the expression of chimeric proteins containing a β1 cytoplasmic domain is, indeed, a reduction of the normal cytoskeleton-integrin connection. Furthermore, the ability to rapidly assay this integrin β1 dominant negative effect via the LPA dependent detachment is a useful system, which will allow for mapping of the sequences within the β-cytoplasmic domain involved in association with the cytoskeleton. The nature of the molecules, which interact with integrins on the cytoplasmic side of the cell remains unclear, despite intensive study. There is in vitro evidence that both talin (Horwitz *et al.*, 1986) and α-actinin (Otey *et al.*, 1990) can bind to β-cytoplasmic peptides. While both of these molecules co-localize with integrins in focal adhesion sites, there is no in vivo evidence that they directly bind to integrins. We believe this experimental system will be a valuable tool in the identification of proteins that interact directly with the β1 cytoplasmic domain in vivo.

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