Contrasting Roles for Integrin $\beta_1$ and $\beta_5$ Cytoplasmic Domains in Subcellular Localization, Cell Proliferation, and Cell Migration

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Abstract. To carry out a detailed comparison of the roles of integrin $\beta_1$ and $\beta_5$ cytoplasmic domains, we expressed both wild type $\beta_1$ and chimeric $\beta_{15}$ constructs in CHO cells. In the latter, the cytoplasmic domain of $\beta_1$ was replaced with that of $\beta_5$. The human $\beta_1$ and $\beta_{15}$ constructs appeared at similar levels at the cell surface (mostly as $\alpha\beta_1$ heterodimers) and contributed equally to CHO cell adhesion to fibronectin. However, $\beta_1$ but not $\beta_{15}$ localized to focal adhesion-like structures when CHO cells were spread on fibronectin. Furthermore, only the $\beta_1$-CHO cells showed increased proliferation in response to fibronectin plus an integrin-activating anti-$\beta_1$ antibody, and showed increased appearance of $^{32}$P-labeled protein (p90) that correlated with proliferation. In sharp contrast, the $\beta_{15}$-CHO cells were notably more migratory than $\beta_1$-CHO cells in a transwell haptotactic migration assay. These results indicate that the $\beta_1$ and $\beta_5$ integrin subunit cytoplasmic domains can translate similar adhesive information into highly contrasting subsequent events. Thus, we have established that "inside-out" and "outside-in" integrin signaling pathways are regulated by fundamentally distinct mechanisms. In addition, we suggest that the same properties of the $\beta_1$ cytoplasmic domain that promote recruitment to visible focal adhesion-like structures may also be conducive to cell proliferation. Conversely, the properties of the $\beta_5$ tail that make it less likely to localize into focal adhesion-like structures may contribute to enhanced cell migration.

The 21 transmembrane $\alpha\beta$ heterodimers in the integrin family of cell adhesion receptors (26, 34) play key roles in two-way signaling across the membrane. The diverse cytoplasmic domain sequences within the various integrin $\alpha$ and $\beta$ subunits are particularly important in this regard.

In a process known as "inside-out signaling," constitutive cellular signals or agonist-triggered signals act through integrin cytoplasmic domains to regulate the ligand-binding properties of the extracellular domains (23, 76). Experiments involving deletions and point mutations within the cytoplasmic domains of $\beta_1$ (25), $\beta_2$ (31, 32), and $\beta_3$ (12) have focused attention on a few conserved residues in each of these subunits as being most critical for control of cell adhesion.

Conversely, external ligand binding may cause changes in cytoplasmic domains (outside-in signaling) associated with a diversity of postligand binding events, such as cell spreading, migration, differentiation, focal adhesion formation, and gene induction (9, 13, 34, 43). Thus far, despite differences in $\beta$ subunit cytoplasmic domain sequences, many integrin-mediated signaling events appear to be rather non-specific. For example, several different integrins are able to cause calcium flux (37, 58, 60, 65, 70, 86), a rise in intracellular pH (36, 45, 71, 72), and activation of $\gamma$25$^{\alpha\beta}$k (24, 42, 47). For some postligand binding events, resulting from outside-in signaling through integrins, it is clear that cytoplasmic domains do play specific roles. For example, chimeric $\alpha$ chain constructs were used to demonstrate that the $\alpha_1$ and $\alpha_4$ cytoplasmic domains differed from the $\alpha_2$ cytoplasmic domain with respect to collagen gel contraction and cell migration mediated by $\alpha_2\beta_1$ (VLA-2) (9).

An obvious assumption has been that the subcellular localization of integrins plays a key role in determining their functions. For example, integrin clustering may make an important contribution to cell adhesion (30). In highly spread cells, a very prominent type of integrin cluster called a focal adhesion complex has been studied in great detail. A variety of studies involving deletions (25, 49, 64, 75), chimeras (21, 43), alternatively spliced $\beta_1$ (4), and point mutations (64) have established that the $\beta_1$ cytoplasmic domain controls $\beta_1$ integrin localization to focal adhesions. Many of the cytoplasmic domain residues critical for focal adhesion localization (64) are shared by several other $\beta$ subunits, thus explaining why the cytoplasmic domains of $\beta_1$ and $\beta_3$ have similar capability to localize to focal adhesions (74). Despite this abundance of background information, the functional consequences of having integrins that differ in their ability to localize to focal adhesions has only begun to be explored (68). For example, among the integrin $\beta_1$, $\beta_3$, $\beta_5$, and $\beta_6$ cytoplasmic do-
mains, the latter has the most distinctive primary amino acid sequence (61, 88). Perhaps related to this, the $\alpha'\beta$ and $\alpha'\beta_2$ integrins appear to differ in terms of localization to focal adhesions and contribution to cell migration (44, 82). However, these reported functional differences between $\alpha'\beta_2$ and $\alpha'\beta$, have not yet been mapped to specific domains; (e.g., $\beta$ chain cytoplasmic domains).

Here, we have expressed in CHO cells cDNA constructs coding for human f$\alpha$, and a chimeric $\beta_1$-s, subunit, in which the cytoplasmic domain of $\beta_1$ was replaced with that of $\beta_5$. These have enabled us to carry out highly controlled and detailed comparative studies showing that (a) the $\beta_1$ and $\beta_5$ cytoplasmic domains differed dramatically in their control of integrin subcellular localization, and (b) that differences in subcellular localization were associated with differences in integrin-dependent proliferation and migration but not cell adhesion. Thus, mechanisms for cytoplasmic tail control of cell adhesion (inside-out signaling) are fundamentally different from mechanisms for control of diverse postadhesion events (outside-in signaling).

Materials and Methods

Cells, Transfections, and Cell Culture

CHO cells negative for the dihydrofolate reductase gene (dhfr-)$^3$ were grown in MEM $\alpha$ medium with 10% FCS, and then switched to MEM $\alpha'$ with 10% dialyzed FCS (SRH Biosciences, Lexena, KS) after transfection. The dhfr$^+$ p901 vector (40) was kindly provided by Dr. M. Rosai, Biogen Co. (Cambridge, MA). The pECE vector containing the $\beta_1$ insert has been previously described (22). A fragment corresponding to the $\beta_5$ cytoplasmic domain was obtained from mRNA (from the CCL-228 carcinoma line) by reverse transcription PCR. In one of the oligonucleotide primers used for PCR, a single mismatch was incorporated to create a HindIII site at the carboxy-terminal side of the transmembrane region. At this site, coding for the amino acids Lys and Leu, AAGCTG was convened to AAGCTCA (a silent mutation). The entire PCR-derived $\beta_5$ insert was subcloned into pBluescript (Stratagene, La Jolla, CA) and sequenced. The $\beta_5$ cytoplasmic domain was removed from the pECE vector using HindIII/XbaI sites (just after the transmembrane and in the 3' polylinker region, respectively) and replaced with the $\beta_5$ cytoplasmic domain also excised (from pBluescript) from the amino acids Lys and Leu, AAGCTG was convened to AAGCTCA (a silent mutation). At this site, coding for the amino acids Lys and Leu, AAGCTG was convened to AAGCTCA (a silent mutation). The latter was transfected with $1001$ dhfr $^+$ plasmid plus $\beta_5$ cDNA in the pRc/CMV vector (Invitrogen, San Diego, CA), but during methotrexate amplification (in parallel with $\beta_1$ and $\beta_15$ amplification), the $\beta_5$ expression was lost.

Antibodies and Extracellular Matrix (ECM) Proteins

The monoclonal antibodies used were: A-1A5 (28), TS2/16 (27), and 4B4 (52), all anti-human $\beta_1$; PBI, anti-hamster $\alpha'\beta_1$ (7); 7E2, anti-hamster $\alpha'\beta_2$ (18); B1E5, anti-$\alpha'2$ cross-reactive with human and hamster (83); and 1O6, anti-$\beta_2$ cross-reactive with human and hamster (59). Rabbit polyclonal sera to $\beta_5$ (61) and $\alpha'$ (35) cytoplasmic domain peptides were previously described. Both fibronectin and vitronectin were purchased from Telios Pharmaceuticals (La Jolla, CA), and pronectin, a synthetic Arg-Gly-Asp-containing matrix protein, was obtained from Promega Corp. (Madison, WI). A mouse monoclonal antibody to the carboxy-terminal peptide was obtained from Sigma Immunochemicals (St. Louis, MO), and the mouse anti-phosphotyrosine antibody 4G10 (16) was a gift from Dr. Dan FCulen (FACScan®; Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Flow Cytometry

Cells were incubated in PBS containing 1% BSA (Gibco Laboratories, Grand Island, NY) for 30 min on ice. Then washed three times in the same solution. Subsequently, cells 2.3 x 10$^5$ were incubated with purified antibodies (1-10 $\mu$g/ml) or ascites (diluted 1:100) for 45 min, and then washed three times in PBS containing 1% BSA, and treated with goat anti-mouse IgG coupled to fluorescein (Cappel Laboratories, Cochranville, PA) for 45 min on ice. After three washes, cells were resuspended in PBS and analyzed using a flow cytometer (FACSscan®; Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Radiolabeling and Immunoprecipitation

Cells were surface labeled with $^{35}$S methionine and cysteine, and then lysed in the presence of 1% NP-40 (Sigma Chemical Co., St. Louis, MO), and pronectin, a synthetic Arg-Gly-Asp-containing matrix protein, was obtained from Promega Corp. (Madison, WI). Immunoprecipitations were performed using protein A-Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) previously incubated with unlabeled cell extract to prevent nonspecific binding of radiolabeled material. Labeled extracts were precleared by incubation with normal mouse or rabbit serum, followed by Staphylococcus aureus Cowan 1 (Pansorbin; Calbiochem Corp.). Immunoprecipitations were performed using monoclonal antibodies at 2 $\mu$g/ml (purified) or diluted 1:100 (ascites), with 3 x 10$^6$ cpm of radiolabeled cell extract per sample. Rat anti-mouse IgG mAb 1871 (87) was added to optimize binding to protein A-Sepharose. Incubations lasted from 30 min to overnight, and then samples were washed three times in 1% NP-40 and analyzed by SDS-PAGE using 6-8% acrylamide gels under non-reducing conditions, unless otherwise stated.

Adhesion and Migration Assays

Cell adhesion assays were performed in 96-well microtiter plates (Flow Laboratories Inc., McLean, VA) coated overnight at 4°C with extracellular matrix protein ligands, followed by 0.1% BSA (for 30 min at 37°C) added to block nonspecific adhesion. Cells were labeled by incubation with the fluorescent dye BCECF (Molecular Probes, Inc., Eugene, OR) for 20 min. After washing twice with PBS, 5 x 10$^4$ of the labeled cells were added in triplicate to plates in the presence or absence of antibodies (1 $\mu$g/ml or 50 $\mu$g/ml of hybridoma supernatant) and incubated for 20 min at 37°C, followed by three washes with RPMI medium containing 0.1% BSA. Cells remaining attached to the plate were analyzed using a fluorescence concentration analyzer (IDEXX Co., Portland, ME). Results are reported as the mean ± 1 SD.

Haptotactic migration assays were performed as described (44), using Boyden chambers (Transwell; Costar Corp., Cambridge, MA), containing membranes with 6.5 mm diameter, 10 $\mu$m thickness, and $8 \mu$m pore size. The lower faces of the membranes were coated overnight with fibronectin, vitronectin, or pronectin (Promega Corp.) at 3 $\mu$g/ml in serum-free medium. Cells were labeled with $^{35}$S methionine for 3 h, washed, and then incubated for 10-15 min on ice with mAb PBI to block hamster $\alpha'\beta_1$ function. In some experiments, cells were also preincubated with TS2/16 (1 $\mu$g/ml). For migration assays, the upper and lower faces of the membrane were equilibrated in $\alpha'$ MEM with 2% FCS, and then the cells were placed in the upper compartment (50,000/cameral, and incubated at 37°C for 1-2 h. After extensive washing, membranes were removed and analyzed by liquid scintillation counting, and the mean of triplicate values for $^{35}$S cpm representing each experimental condition was calculated. No radioactivity above background was detected in the upper part of the chambers after washing.
**Immunofluorescence**

Cells were detached from tissue culture dishes using 2.5 mM EDTA in PBS, washed in serum-free α-MEM, and resuspended in the same medium containing 1% FCS. Then cells were plated on fibronectin-coated slides and incubated for 6 or 12 h. After adhesion and spreading, nonadherent cells were removed and the adherent cells were fixed for 15 min on ice with 0.5% paraformaldehyde. After three washes with PBS, methanol was added for 2 min, followed by four more washes. Fixed cells were then incubated for 30-40 min with anti-β mononcational antibody A-IA5 (I:400), antiallum monoconal antibody (1:500 dilution of ascites; Sigma Immunochemicals), anti-hamster αβ; PBI (1 μg/ml), or anti-P-Tyr (1 μg/ml), then washed and incubated with FITC-labeled goat anti-mouse antibody (1:40 dilution; Calbiochem Corp.) for 35 min. After staining, slides were washed with PBS four times and mounted.

**Proliferation Assays**

Cells were serum deprived by culturing in α-medium containing 1% FCS for 24 h, and then 50,000 cells were placed in the same solution, in 96-well plates coated with or without different ECM proteins. Also, in some experiments, cells were preincubated with various antibodies for 15 min before they were added to the 96-well plates.

Proliferation was measured by pulsing the cells with 1 μCi [3H]thymidine/well during the last 8 h of 36-h culture. As a positive control, cells were plated in the presence of 10% FCS. A PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA) was used to transfer [3H]-labeled DNA to glass fiber filters for analysis by liquid scintillation counting.

Alternatively, cell proliferation was assessed using the MTT dye method, which detects mitochondrial enzymes (79). Briefly, during the last 4 h of 36-h culture at 37°C, MTT (3-[4,5-mightylthazol-2-yl]-2,5-mphenyltetrazolium bromide) was added to each well to give 0.5 mg/ml. Then, after the cells were solubilized in 0.01 N HCl containing 10% SDS for 8-12 h at 37°C, OD at 650 minus 590 nm was determined using a microplate reader (VMAx; Molecular Devices Co.).

**Results**

**Expression of Human β1, and β15 cDNA in CHO Cells**

The expression of human β1 and β15 cDNA in CHO cells was evaluated by flow cytometry as shown in Fig. 1. After selection in nucleoside-free medium, human β1 and β15 were expressed on CHO cells at comparable levels (column 3) that were at least as high as the level of endogenous hamster β1 (column 2) and greater than the level of staining with a negative control antibody (column 1). After amplification in methotrexate followed by cell sorting, human β1 and β15 were homogeneously expressed at high levels (column 4) 10-fold greater than endogenous hamster β1 in the same cells (column 2). Mock-transfected CHO cells (top row) expressed endogenous hamster β1, but showed no cross-reactivity with the anti-human β1 antibody. Levels of hamster β1 did not change (column 5), but hamster α2 was expressed at equivalently higher levels on β1-CHO and β15-CHO cells compared to control CHO cells (column 6). Consistent with this, others have noted that β1 on CHO cells is predominantly associated with the α2 subunit (69).

To avoid selection for anomalous functional traits, no efforts were made to establish clonal cell lines. Instead, bulk cell populations amplified for β1 or β15 expression were used for all subsequent experiments, along with untransfected or mock-transfected CHO cells.

The human β1 and β15 subunits expressed at the surface of CHO cells were further analyzed by immunoprecipitation from nonionic detergent cell extracts of 121-I-labeled polyclonal serum to the β1 cytoplasmic domain immunoprecipitated the 90-kD hamster β1 subunit (lower arrow), presumably associated with hamster α2 from β1-CHO, β15-CHO and CHO-MTX cells (Fig. 2 A, lanes a-c). An additional band corresponding to the 100-kD chimeric β15 chain was seen only from β15-transfected cells (lane b, upper arrow). As expected, an antibody to the human β1 ectodomain (A-IA5) coinmunoprecipitated associated β1 and α subunits from β1 CHO (lane d) and β1-CHO cells (lane e), but not from mock-transfected (lane f) or untransfected CHO cells (not shown). The pattern of bands recognized by the anti-human β1 antibody (lanes d and e) looked very similar to the α2 and β1 bands immunoprecipitated from β1-CHO (lane g), β15-CHO (lane h), and CHO-MTX (lane i) by an antibody recognizing the cytoplasmic domain of hamster α2. These results are consistent with α2 being the predominant subunit associated with human or hamster β1 on these cells.

Immunoprecipitation from 35S-metabolically labeled β15-CHO cells confirmed the presence of the β15 protein (Fig. 2 B). Antiserum to the β15 carboxy terminus recognized an extra band migrating similar to β1 (lane b, arrow) that was...
Adhesive Properties of β1-CHO and β1/5-CHO Cells

The adhesion of β1 and β1/5 CHO cells to fibronectin (Fig. 3A) was carried out both in the absence (parts a and b) and presence (parts c and d) of the PBI antibody added to block background adhesion mediated by hamster α2β1. In either case, adhesion by β1 and β1/5 CHO cells was greater than that seen by control (CHO-MTX) cells (parts a and c), although the difference was more obvious when PBI was present (part c). When the anti-human β1 antibody 4B4 was added, adhesion by β1 and β1/5 CHO cells was no longer elevated compared to the CHO-MTX cells (parts b and d). The inhibitory effect of 4B4 was especially obvious when PBI was also present (part d). Thus, the substantial contributions of human β1 and β1/5 subunits to fibronectin adhesion were most obvious when the background contribution of hamster α2β1 was blocked.

In contrast to fibronectin, collagen IV and laminin supported only low levels of adhesion by the various CHO cells, and the adhesion by β1- and β1/5-CHO cells was not significantly greater than adhesion by CHO-MTX cells (Fig. 3B, parts e and f). These results are consistent with β1 being mostly associated with the hamster α2 subunit. Also, adhesion to vitronectin was essentially unchanged among CHO, β1-CHO, and β1/5-CHO cells (not shown), indicating that the presence of the β1/5 chimera had no obvious effect on the adhesive function of hamster α2β1.

In the presence of PBI (anti-hamster α2β1), both β1-CHO and β1/5-CHO cells bound equally well to fibronectin in a dose-dependent fashion, markedly above the level of adhesion seen for mock-transfected (CHO-MTX) cells (Fig. 3B). In the presence of the stimulatory anti-β1 antibody TS2/16, adhesion of both β1- and β1/5-CHO cells was upregulated to the same extent, especially at the lower doses of fibronectin. In addition, titration of Mg2+ (from 0.005 to 50 mM) revealed no significant differences between β1- and β1/5-dependent adhesion (Fig. 3C). In summary, the results in Fig. 3 show that the β1 and β1/5 subunits have a similar level of constitutive activity, are equally responsive to stimulation by the anti-β1 antibody TS2/16, and show equivalent use of Mg2+ to support adhesion.

Subcellular Localization of β1/5 and β1 in CHO Cells

To compare the subcellular distribution patterns of the β1 and β1/5 subunits, β1- and β1/5-CHO cells were plated for 6–12 h on fibronectin. Then, after fixing and staining of permeabilized cells with the anti-β1 antibody A-1A5 (specific to the extracellular domain), a very distinct distribution pattern was observed for the two cell lines (Fig. 4). Wild type human β1 (Fig. 4, A, C, E, and G) consistently showed a punctate distribution pattern with many focal adhesion-like structures present. In marked contrast, β1/5 was diffusely distributed (Fig. 4, B, D, F, and H) and not localized to focal adhesion-like structures in these and numerous other experiments. Control experiments (Fig. 5) showed that both β1-CHO cells (A, C, and E) and β1/5-CHO cells (B, D, and F), had equal ability to form focal adhesions containing hamster α2β1 (weakly stained; A and B), talin (strongly stained; C and D) or phosphotyrosine (E and F). Antibody staining of nonpermeabilized cells or preincubation with antibody before permeabilization also yielded striking differences in the localization patterns of β1 and β1/5, with the latter being much more diffuse (not shown). These control experiments indicate that the diffuse staining seen for β1/5 was not simply caused by selective accumulation of that protein within intracellular pools. Also, endogenous wild type hamster β1 remained diffusely localized in both β1- and β1/5-CHO cells, consistent with the diffuse localization of β1/5.

Contribution of β1 and β1/5 to CHO Cell Proliferation

There have been several reports that integrins can deliver
Figure 3. Comparison of adhesive properties of $\beta_1$-CHO, $\beta_{1,5}$-CHO, and CHO-MTX cells. (A) Plastic surfaces were coated with fibronectin, collagen IV, or laminin (each at 5 $\mu$g/ml), and then cell adhesion assays were carried out as described in Materials and Methods. The fibronectin adhesion assays were carried out in the presence of control antibody, anti-human $\beta_1$ (4B4), anti-hamster $\alpha^5\beta_1$ (PB1), or both 4B4 and PB1 as indicated. (B) Adhesion to fibronectin (coated at 0.5, 2.0, and 10 $\mu$g/ml) was carried out in the presence of 2 $\mu$g/ml purified PB1 (to block hamster $\alpha^5\beta_1$ function). As indicated, 1 $\mu$g/ml of stimulatory anti-human $\beta_1$ mAb TS2/16 was added for some assays. (C) Adhesion to fibronectin (coated at 5 $\mu$g/ml) was carried out in the presence of varying concentrations of Mg$^{2+}$, and a constant level of PB1 (2 $\mu$g/ml).

costimulatory (15, 50) or direct stimulatory (53, 80) signals leading to cell proliferation. We hypothesized that the striking differences in the subcellular localization properties of $\beta_1$ and $\beta_{1,5}$ might correlate with differences in their contributions towards proliferation. Thus, conditions were set up to examine the contribution of integrins towards proliferation of CHO cells, exploiting the window of time (20–36 h) in which the CHO cells could maintain viability while be-
Figure 4. Immunofluorescent localization of human $\beta_1$ and $\beta_{1,5}$ in CHO cells. The $\beta_1$-CHO (left panels) and $\beta_{1,5}$-CHO cells (right panels) were allowed to attach and spread on fibronectin-coated slides for 6 h (A-D) or 12 h (E-H), and then were stained with anti-human $\beta_1$ mAb A-IA5 as described in Materials and Methods. x900 (A and B), x630 (C-F), or x400 (G and H).
Figure 5. Immunofluorescent localization of hamster αβ1, talin, and phosphotyrosine in β1- and β13-CHO cells. The β1-CHO (left panels) and β13-CHO cells (right panels) were allowed to attach and spread on fibronectin-coated slides for 6 h, and then were stained with anti-hamster αβ1 mAb PBI (A and B), antitalin polyclonal antiserum (C and D), or anti-phosphotyrosine antibody (E and F) as described in Materials and Methods. ×630 (A–D) and ×400 (E and F).
ing deprived of serum. As shown in Fig. 6, the combination of immobilized fibronectin plus soluble TS2/16 caused a marked proliferation of serum-deprived \( \beta_1 \)-CHO cells, but not \( \beta_1/5 \)-CHO or control CHO-MTX cells, as measured by uptake of \(^{3}H\)thymidine. Notably, neither fibronectin nor TS2/16 alone was sufficient. Also, the proliferative response to TS2/16 and fibronectin was not appreciably blocked by anti-hamster \( \alpha_5 \beta_1 \) antibody PB1, indicating that fibronectin, like TS2/16, was acting through the human \( \beta_1 \) subunit. In control experiments, a nonstimulatory anti-human \( \beta_1 \) antibody (4B4), the hamster \( \alpha_5 \beta_1 \) (PB1), and the control antibody P3 had no stimulatory effect on proliferation, either alone or together with fibronectin. In the presence of 10% serum, transfected and control CHO cells all proliferated at a high level, with no detectable difference among them.

Interestingly, intact fibronectin could not be replaced by the 120-kD fibronectin fragment, even though it contains both the central \( \alpha_5 \beta_1 \) binding site (the RGD region), as well as a second synergizing site of interaction with \( \alpha_5 \beta_1 \) (54). The requirement for intact fibronectin was not unique to CHO cells; a human B cell line (JY) and a myeloid cell line (KG1) also showed a similar \( \beta_1 \)-dependent proliferation to intact fibronectin, but not to FN-40 or to FN-120 fragments (not shown).

To further emphasize the proliferation difference between \( \beta_1 \)-CHO and \( \beta_1/5 \)-CHO cells, the results of six different \(^{3}H\)thymidine uptake experiments are shown in Table I. In each experiment, the proliferation of \( \beta_1 \)-CHO cells was markedly greater than \( \beta_1/5 \)-CHO cells in response to a combination of FN plus TS2/16. Such a difference was not obvious if the cells were not stimulated or stimulated with FN alone (Table I). In general, MTT staining of CHO and \( \beta_1/5 \)-CHO cells was not diminished as a result of FN plus TS2/16 treatment, and \( \beta_1 \)-CHO and \( \beta_1/5 \)-CHO cells showed no loss of viability when those cells were monitored by MTT staining before.

![Figure 6. Comparison of proliferative responses of \( \beta_1 \)-CHO and \( \beta_1/5 \)-CHO cells.](image-url)

Table I. Proliferation of \( \beta_1 \)- and \( \beta_1/5 \)-CHO Cells Monitored by \(^{3}H\)Thymidine

| Experiment | FN | FN + TS2/16 | CHO-MTX |
|------------|----|-------------|---------|
| 1. \( \beta_1 \)-CHO | -500 | 3,000 | 27,500 | 11,000
| \( \beta_1/5 \)-CHO | -200 | 3,200 | 2,400 |
| 2. \( \beta_1 \)-CHO | -140 | 2,500 | 30,600 | 10,500
| \( \beta_1/5 \)-CHO | -180 | 2,000 | 2,800 |
| 3. \( \beta_1 \)-CHO | 1,500 | 4,600 | 10,000 | 13,700
| \( \beta_1/5 \)-CHO | 2,400 | 3,200 | 5,500 |
| 4. \( \beta_1 \)-CHO | -200 | 3,800 | 25,000 | 12,000
| \( \beta_1/5 \)-CHO | -5,800 | 1,400 | 1,600 |
| 5. \( \beta_1 \)-CHO | 1,100 | 1,600 | 19,600 | 10,150
| \( \beta_1/5 \)-CHO | -3,400 | 2,600 | -1,800 |
| 6. \( \beta_1 \)-CHO | 1,100 | 2,300 | 7,500 | 8,700
| \( \beta_1/5 \)-CHO | 1,600 | 1,200 | 2,900 |

* All numbers represent cpm of \(^{3}H\)thymidine and are the mean of six determinations.

Table II in three separate experiments, \( \beta_1 \)-CHO cells but not \( \beta_1/5 \)-CHO cells proliferated in response to a combination of FN plus TS2/16. Consistent with results in Table I and Fig. 6, elevated proliferation by \( \beta_1 \)-CHO cells was not obvious if the cells were not stimulated or stimulated with FN alone (Table II). In general, MTT staining of CHO and \( \beta_1/5 \)-CHO cells was not diminished as a result of FN plus TS2/16 treatment, and \( \beta_1 \)-CHO and \( \beta_1/5 \)-CHO cells showed no loss of viability when those cells were monitored by MTT staining before.
Table II. Proliferation of $\beta_1$- and $\beta_{15}$-CHO Cells Monitored by MTT Staining

| Experiment | No stimuli | FN | FN + TS2/16 |
|------------|------------|----|-------------|
| 1. CHO-MTX | 0.24       | 0.13 | 0.29 |
| $\beta_1$-CHO | 0.19       | 0.27 | 1.11 |
| $\beta_{15}$-CHO | 0.27      | 0.21 | 0.24 |
| 2. CHO-MTX | 0.38       | 0.40 | 0.34 |
| $\beta_1$-CHO | 0.41       | 0.29 | 1.27 |
| $\beta_{15}$-CHO | 0.31      | 0.39 | 0.18 |
| 3. CHO-MTX | 0.34       | 0.39 | 0.23 |
| $\beta_1$-CHO | 0.31       | 0.35 | 0.98 |
| $\beta_{15}$-CHO | 0.39      | 0.26 | 0.24 |

* In each experiment, cells were serum-deprived for 24 h and then cultured for 26 h in the presence or absence of immobilized FN and soluble TS2/16 as described (see Materials and Methods).

† All numbers represent OD570 values indicative of MTT staining of viable cells (see Materials and Methods), and they are the mean of triplicate determinations.

during, and after a 32-h interval of serum deprivation (not shown). Thus, the proliferation differences observed in Fig. 6 and Tables I and II were not caused by selective apoptosis.

Specific Phosphorylation of a 90-kD Protein in $\beta_1$-CHO Cells

The same conditions that caused a difference in proliferation between $\beta_1$-CHO and $\beta_{15}$-CHO cells also yielded an obvious biochemical difference. The $\beta_1$-CHO and $\beta_{15}$-CHO cells were stimulated with fibronectin and TS2/16 while in the presence of $[^{32}P]$orthophosphate, and then whole cell extracts were prepared in the presence of phosphatase inhibitors and analyzed by SDS-PAGE. Whereas several phosphoproteins were present in both $\beta_1$-CHO and $\beta_{15}$-CHO cell extracts, a 90-kD phosphoprotein band was present only in the extract from $\beta_1$-CHO cells (Fig. 7). Using a common 95-kD phosphoprotein as an internal standard, gel scanning confirmed that the p90 was prominent in $\beta_1$-CHO cell extracts, but almost undetectable in extracts from $\beta_{15}$ cells (Fig. 7), CHO cells, or CHO-MTX cells (not shown).

In several additional experiments, the specific appearance of the p90 phosphoprotein showed a strong correlation with cell proliferation (Table III). Under conditions of serum deprivation, the p90/p95 ratio was elevated for $\beta_1$-CHO but not $\beta_{15}$-CHO cells, and only when both fibronectin and TS2/16 were present at the same time. In contrast to TS2/16, an anti-\(\beta_1\) antibody not able to stimulate cell adhesion (4B4) did not costimulate (with fibronectin) the appearance of either p90 phosphoprotein or proliferation. Under normal growth conditions (10% serum), both proliferation and elevated p90 expression were observed. In summary, the appearance of p90 is closely associated with cell proliferation and provides an independent readout consistent with the selective involvement of $\beta_1$ but not $\beta_{15}$ in cell proliferation.

| Table III. Correlation between p90 Phosphorylation and $\beta_1$-CHO Proliferation |
|----------------------------------|------------------|------------------|
| Growth Condition | Cells | p90/p95 ratio | Proliferation* |
| 1% S | $\beta_1$-CHO | 0.08 | - |
| | $\beta_{15}$-CHO | 0.11 | - |
| 1% S; TS2/16 | $\beta_1$-CHO | 0.19 | - |
| | $\beta_{15}$-CHO | 0.14 | - |
| 1% S; TS2/16; FN | $\beta_1$-CHO | 0.65 | + |
| | $\beta_{15}$-CHO | 0.12 | - |
| 1% S; 4B4; FN | $\beta_1$-CHO | 0.08 | - |
| | $\beta_{15}$-CHO | 0.12 | - |
| 10% S | $\beta_1$-CHO | 0.72 | + |
| | $\beta_{15}$-CHO | 0.68 | + |

* Proliferation of $\beta_1$-CHO and $\beta_{15}$-CHO cells was determined as described in the legend for Fig. 6. +, >10-fold greater $\beta_1$ proliferation compared to $\beta_{15}$ after subtraction of CHO-MTX background; -, less than twofold greater proliferation for $\beta_1$-CHO cells.

† Ratios of the levels of phosphorylated p90 relative to p95 were based on peak areas obtained from gel scans such as shown in Fig. 7.

Migration on Fibronectin

The $\alpha^2\beta_1$ integrin has a well-established role in cell migration on fibronectin. Thus, to further investigate the functional properties of the $\beta_1$ and $\beta_{15}$-CHO cells, we used modified Boyden chambers containing 8.0 $\mu$m porous membranes to analyze cell migration. In the presence of PBI (added to block the adhesion of endogenous hamster $\alpha^2\beta_1$)
β1-CHO cells showed only background cell motility towards fibronectin, vitronectin, pronectin, and towards surfaces not specifically coated with any matrix protein. Because the migration of β1-CHO cells was not significantly elevated or depressed compared to untransfected or mock-transfected CHO cells (Fig. 8 A), human β1 did not appear to exert either a positive or negative effect on CHO cell migration. In contrast, β1,5-CHO cells displayed markedly increased migration toward a fibronectin source (Fig. 8 A), and even a higher level of migration in the presence of the anti-β1 stimulating antibody TS2/16. The presence of TS2/16 alone had no effect on cell migration if ECM protein was omitted. In separate experiments, cells crossing the membrane towards the lower compartment were counted by microscopic examination, yielding data comparable to that obtained using 35S-labeled cells (not shown). The difference in migration between β1-CHO and β1,5-CHO cells was maintained for ≈12 h (Fig. 8 C). These results point to a pronounced difference in the contribution of the β2 and β1 cytoplasmic domains towards cell migration.

Analysis of additional experimental conditions revealed that migration of β1,5-CHO cells was markedly elevated in the presence of 10% serum, compared to 0.1% serum (Fig. 8 B). This serum effect could not be mimicked by adding PMA or PDGF. Also, the presence of 10% serum did not support nonspecific migration when a control ligand (collagen) was used.

Discussion

The central finding in this paper is that the β1 and β3 cytoplasmic domains were equally able to support cell adhesion, but showed marked differences in multiple postligand binding functions. Whereas the β1 cytoplasmic domain was better suited for localizing into focal adhesion-like structures, and selectively contributed to cell proliferation (associated with p90 phosphorylation), the β3 cytoplasmic domain facilitated migration of CHO cells on fibronectin.

Differences in Subcellular Localization

Previously, it was found that αβ2 and αβ3 had different subcellular distribution patterns in cells spread on vitronectin (82), but it was not shown that the β chain cytoplasmic domains were responsible. Here, we provide explicit evidence that replacement of only the cytoplasmic domain of
with that of $\beta_3$ was sufficient to alter dramatically the diffuse state. Approximately 10 key residues within the $\beta_1$ cytoplasmic domain have been identified that determine whether $\beta_1$ will localize to focal adhesions or will be distributed more diffusely (64). Three of these residues in $\beta_1$ (Fig. 9, positions 28, 35, and 38) are missing in $\beta_3$, thus perhaps explaining why $\beta_1$ fails to localize to focal adhesions. Related to this, the same three $\beta_1$ residues (positions 28, 35, and 38) occur within a potential $\alpha$-actinin binding region (57) that may be important for focal adhesion formation. Attention is now also focused on the 10 additional residues that make $\beta_3$ longer than $\beta_1$, (Fig. 9), which could potentially exert a negative effect.

**Similarities in Cell Adhesion**

The $\beta_1$ and $\beta_{13}$ subunits made virtually identical contributions towards CHO cell adhesion regardless of fibronectin levels or divalent cation levels, and both were equally responsive to stimulation by an anti-$\beta_1$ activating antibody (TS2/16). Because of these nearly identical cell adhesion results, we consider it highly likely that the differences we observed in other functions were not simply caused by a gross alteration of the overall integrin structure. More importantly, the outside-signaling mechanisms regulating the constitutive adhesive activity of $\beta_1$ integrin in CHO cells appear to be independent of the mechanisms regulating subcellular localization, cell migration, or cell proliferation. This leads to an important qualitative distinction between the type of integrin clustering that may facilitate cell adhesion in a short-term assay of 0.5-1 h (30) and the integrin clustering that is visible in focal adhesions after 6-12 h.

Key residues within the $\beta_1$ (31) and $\beta_1$ (12) cytoplasmic domains have been identified that are involved in the regulation of integrin-ligand adhesive interactions. For the most part, those residues are conserved in both $\beta_1$ and $\beta_3$ (Fig. 9, positions 30-32, 38, 39) if you allow the phenylalanine at position 39 in $\beta_3$ to substitute for the Tyr at position 38 in $\beta_1$. Thus, a common mechanism for controlling cell adhesion/ligand binding appears to be shared by multiple integrin $\beta$ subunit tails, even though they may differ markedly in other functional respects.

**Differences in Cell Proliferation**

A large body of literature (reviewed in references 33 and 46) has addressed the effects of fibronectin and other ECM proteins on cell proliferation. These ECM proteins act at least partially through integrins, because many integrin ligands or anti-integrin antibodies had costimulatory effects on cell proliferation (15, 50, 55, 66, 73, 85). Although a few studies have suggested that mitogenesis can be achieved directly through integrins (53, 78, 80), most studies have failed to sort out direct proliferative signals from other events such as integrin-mediated cell adhesion, shape change, and potentiation of other growth triggering agents. Our results now provide a clear demonstration that the $\beta_1$ and $\beta_3$ cytoplasmic domains differ markedly in their ability to influence cell proliferation, even though they support cell adhesion to a similar extent. We hypothesize that the ability of $\beta_3$ to be recruited more readily into macromolecular subcellular structures could facilitate signaling required for cell proliferation. Conversely, the more diffuse localization of the $\beta_1$ cytoplasmic domain might prevent molecular associations needed for proliferation. In this regard, subcellular structures such as focal adhesions are increasingly seen as sites of signaling, where relevant cytoskeletal proteins and other proteins may become exposed to kinases and/or phosphatases (24, 34, 38, 48).

We consider it unlikely that the observed proliferation differences are caused by an alteration in the pattern of associated $\alpha$ chains between $\beta_1$ and $\beta_{13}$, because (a) the major $\alpha$ subunit associated with $\beta_1$ on CHO cells (i.e., $\alpha_2$) was expressed at identical levels in $\beta_1$- and $\beta_{13}$-CHO cells; (b) both $\beta_1$ and $\beta_{13}$ yielded identical immunoprecipitation patterns; and (c) there was no evidence for other $\beta_1$-associated $\alpha$ chains, based on negative collagen and laminin adhesion assays and immunoprecipitation results. Furthermore, all available evidence indicates that integrin $\alpha \beta$ pairing specificity is determined by extracellular (and perhaps also transmembrane), but not cytoplasmic domains (5, 6, 14, 20, 56, 74).

Because both TS2/16 and intact fibronectin together were required to trigger proliferation of $\beta_1$-CHO cells, we hypothesize that TS2/16 strengthens the affinity of $\alpha \beta$ for fibronectin (as previously described [3, 8, 19, 81]) thus allowing the integrin, along with proteins interacting with other domains in the intact fibronectin, to be gathered into the appropriate membrane-spanning signaling complex. Alternatively, the anti-$\beta_1$ antibody itself could be sending a costimulatory signal that acts synergistically with intact fibronectin, but not fibronectin fragments or peptides.

In the many previous studies showing cell proliferation as a result of costimulatory signals acting through $\beta_1$ integrins (10, 15, 50, 55, 73, 85), it was often not clear whether the contribution of the $\beta_1$ integrin went beyond simply providing cell adhesion. Our results now indicate clearly that adhesive capability alone is not sufficient (such as in $\beta_{13}$-CHO cells), and that the $\beta_1$ cytoplasmic domain is serving additional signaling and/or localization functions required for cell proliferation.

The nearly all-or-none appearance of phosphorylated p90 in response to fibronectin plus TS2/16 showed a strong correlation with enhanced cell proliferation. At the present time, we cannot determine whether this event is an essential part of the downstream signaling pathway leading to proliferation or part of a divergent pathway. We suspect that p90 is phosphorylated on serine or threonine because it was readily detected at a high level in a whole cell extract, and it was not phosphorylated on the cytoplasmic domain...
seen with antiphosphotyrosine antibody. Among known proteins of ~90 kD, such as HSP-90 (84), PKC-ε (67), and pp90^rsk (11), that are phosphorylated on serine or threonine, pp90^rsk has the most obvious association with cell proliferation (11). However, we found no difference in the level of phosphorylated pp90^rsk immunoprecipitated from β1-CHO and β5-CHO cells, regardless of the stimulation of those cells with fibronectin and TS2/16 (not shown). Thus at present, the identity of our pp90 protein is unknown. Nonetheless, its phosphorylation is closely correlated with cell proliferation, and it provides an independent assay indicating that the β1 tail has a unique signaling function not seen with the β3 tail.

**Differences in Cell Migration**

We hypothesize that differences between the β1 and β3 cytoplasmic domains with respect to subcellular localization are likely to be closely associated with differences in cell migration. For example, the β1 cytoplasmic domain, by promoting more diffuse subcellular localization, could thus facilitate cell migration. In this regard, others have also noted an inverse correlation between focal adhesion formation and cell motility (1, 17, 63). Although α2β1 can have a negative effect on random cell migration when overexpressed in CHO cells (22), our β1-CHO cells did not migrate any less than untransfected or mock-transfected CHO cells in a haptotactic migration assay. Thus, rather than the β1 tail having a negative effect, the β1 cytoplasmic domain seems to be playing a positive role.

In the complex multistep process of cell migration, we propose that β1 and β3 tails may not differ in the initial adhesive steps that are required, but rather, they may show critical differences in a later step involving integrin-cytoskeletal interactions at the leading edge of the cell (68). Alternatively, considering that the β1,3-CHO cells were more migratory than β3-CHO cells only when serum was present, the β3 and β1 cytoplasmic domains may differ in their abilities to translate signals resulting from serum stimulation into a migratory phenotype. Another possibility is that processes such as phosphatase-dependent deadhesion, also known to influence cell motility (29), could differentially act on β1 and β3 cytoplasmic domains.

While the precise biochemical mechanism by which the β3 cytoplasmic tail facilitates migration remains to be determined, we do not wish to imply that the β1 tail is never able to form focal adhesion-like structures during cell migration, especially since the transient occurrence of such structures may be an essential step in cell migration (62). Rather, we simply propose that the β1 tail has properties that make it averse to localization into focal adhesions in highly spread cells, but at the same time, contribute to enhanced cell migration.

The independent regulation of cell adhesion and migration has also been observed in studies involving integrin α chain cytoplasmic domains. Whereas the α2, α3, and αv cytoplasmic domains contributed similarly to cell adhesion (9, 39, 41), the α4 cytoplasmic domain was more effective at supporting migration (9).

In another study, β1 but not β3 supported cell migration, even though the latter was more diffusely distributed (44), thus contrasting with the results shown here. Possibly β3 did not support migration in the previous study because serum was not present, a different cell type was used (a pancreatic carcinoma) or a different α chain (α4) was involved (44).

In conclusion, our results support a general scheme in which different integrins use commonly shared mechanisms to control cell adhesion (inside-out signaling), but translate adhesive information into sharply contrasting subsequent events (outside-in signaling), depending on the unique feature of their different cytoplasmic domains. Thus, we predict that other β subunits with even more divergent cytodomains (β6, β8, and β10) will differ from β1 and β3 in several of the same parameters examined here. In addition, our results begin to suggest how subcellular localization of integrins could exert control over diverse functions such as cell proliferation and cell migration. The challenge in the future will be to identify specific biochemical associations for β1 and β3 cytoplasmic domains that are relevant to their specific functions.

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11. Chen, R.-H., and J. Blenis. 1990. Identification on Xenopus $6$ protein kin-
12. Chen, Y. P., I. Djaffar, D. Pidard, B. Steiner, A. M. Cieutat, J. P. Can-
13. Damsky, C. H., and Z. Werb. 1992. Signal transduetion by integrin recep-
21. Geiger, B., D. Salomon, M. Takeichi, and R. O. Hynes. 1992. A chimeric
23. Ginsberg, M. H., X. Du, and E. F. Plow. 1992. Inside-out integrin signal-
24. Guan, J.-L., J. E. Trevithick, and R. O. Hynes. 1991. Fibronectin/integrin
28. Hemier, M. E., C. F. Ware, and J. L. Strominger. 1983. Characterization
29. Hendey, B., C. B. Klee, and F. R. Maxfield. 1992. Inhibition of neutrophil
33. Hynes, R. O. 1990. Fibronectins. Springer-Verlag New York, Inc., New
32. Hibbs, M. L., S. Jakes, S. A. Stacker, R. W. Wallace, and T. A. Springer. 1991. The cytoplasmic domain of the integrin $\beta$ subunit: sites required for binding to intercellular adhesion molecule 1 and the phosphatase-stabilized phosphorylation site. J. Exp. Med. 174:1227-1238.
32. Hibbs, M. L., H. Xu, S. A. Stacker, and T. A. Springer. 1991. Regulation of adhesion to ICAM-1 by the cytoplasmic domain of LFA-1 integrin beta 2 chain. Science (Wash. DC). 251:1611-1613.
33. Hynes, R. O. 1990. Fibroblasts. Springer-Verlag New York, Inc., New York. pp. 1-546.
34. Hynes, R. O. 1992. Integins: versatility, modulation and signalling in cell adhesion. Cell. 69:11-25.
35. Hynes, R. O., E. E. Mancinotto, M. A. Stepp, L. A. Urry, and G. H. Yee. 1989. Integrin heterodimer and receptor complexity in avian and mammalian cells. J. Cell Biol. 109:409-420.
36. Ingber, D. E., D. Prusty, J. J. Frangioni, E. J. Cr BCE, Jr., C. P. Loche, and M. A. Schwartz. 1995. Control of intracellular pH and growth by fibronectin in capillary endothelial cells. J. Cell Biol. 110:1803-1811.
37. Jaconi, M. E., J. M. Theiler, W. Schlegel, R. D. Appel, S. D. Wright, and P. D. Lew. 1991. Multiple elevations of cytosolic-free Ca$^2+$ in human neutrophils initiated by adherence receptors of the integrin family. J. Cell Biol. 112:1249-1257.
38. Jaken, S., K. Leach, and T. Klauck. 1989. Association of type 3 protein kinase C with focal contacts in rat embryo fibroblasts. J. Cell Biol. 108:697-704.
39. Kassner, P. D., and M. E. Hailer. 1993. Interchangeable alpha chain cyto-
plasmic domains play a positive role in control of cell adhesion mediated by VLA-4 $\alpha$-integrins. J. Exp. Med. 178:649-660.
40. Krovatin, R. J., and P. A. Sharp. 1982. Amplification and expression of sequences cotransfected with a modular dihydrofolic reductase comple-
timentary DNA gene. J. Mol. Biol. 150:601-621.
41. Kawaguchi, S., and M. E. Hailer. 1993. Role of the $\alpha$ subunit cytoplasmic domain in regulation of adhesive activity mediated by the integrin VLA-2. J. Cell Biol. 268:16279-16285.
42. Kornberg, L., H. S. Earp, C. E. Turner, C. Prokop, and R. L. Juliano. 1991. Signal transduction by integrins: increased protein tyrosine phos-
phorylation caused by clustering of $\beta$ integrins. Proc. Natl. Acad. Sci. USA. 88:8392-8396.
43. LaFlamme, S. E., S. K. Akizawa, and K. M. Yamada. 1992. Regulation of fibronectin receptor distribution. J. Cell Biol. 117:437-447.
44. Levenson, E. A., C. A. Pasqualini, and D. A. Cheresh. 1992. Requirement of the integrin $\beta$3 subunit for carcinoma cell spreading or migration on vitronectin and fibronogen. J. Cell Biol. 117:1101-1107.
45. Levenson, D. I., M. A. Schwartz, M. Rosenfeld, and D. A. Cheresh. 1993. Integrin $\beta$5- and $\beta$3-mediated endothelial cell migration is trig-
gered through distinct signalling mechanisms. J. Cell Biol. 121:163-170.
46. Levesque, J. F., J. Harfetz, and A. Hafizto. 1990. A mitogenic fibrinogen receptor that differs from glycoprotein Ib-IIIa. J. Biol. Chem. 265:328-333.
47. Lipfert, L., B. Haimovich, M. D. Schaller, B. S. Cobb, J. T. Parsons, and J. S. Bruggen. 1993. Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125$^{56}$ in platelets. J. Cell Biol. 119:905-912.
48. Maher, P. A., E. B. Pasqualie, J. Y. J. Wang, and S. J. Singer. 1985. Phosphotyrosine-containing proteins are concentrated in focal adhesions and intercellular junctions in normal cells. Proc. Natl. Acad. Sci. USA. 82:6576-6580.
49. Marcantonio, E. E., J. Guan, J. E. Trevithick, and R. O. Hynes. 1990. Mapping of the functional determinants of the integrin beta-1 cytoskeletal domain by site-directed mutagenesis. Cell Regul. 1:597.
50. Matsuyama, T., A. Yamada, J. Kay, K. M. Yamada, S. K. Akizawa, S. F. Schlossman, and C. Morimoto. 1989. Activation of CD4 cells by fibronectin and anti-CD3 antibody: a synergistic effect mediated by the VLA-5 fibronectin receptor complex. J. Exp. Med. 170:1133-1148.
51. McLean, J. W., D. J. Vestal, D. A. Cheresh, and S. C. Ikutaary. 1990. cDNA sequence of the human integrin $\beta$3 subunit. J. Biol. Chem. 265:7126-7131.
52. Mortarini, R., A. Gismondi, A. Santoni, G. Parmiani, and A. Anichini. 1992. Role of the $\alpha$2 integrin receptor in the proliferative response of quiescent human melanoma cells to fibronectin. Cancer Res. 52:4499-4506.
53. Nagai, T., N. Yamakawa, S. Aota, S. S. Yamada, S. K. Akizawa, K. Olden, and K. M. Yamada. 1991. Monoclonal antibody characterization of two distant sites required for function of the central cell-binding domain of fibronectin in cell adhesion, cell migration, and matrix assembly. J. Cell Biol. 110:1343-1344.
54. Pasqualini, R., J. Bodorova, S. Ye, and M. E. Hailer. 1993. Study of the
structure, function and distribution of β1 integrins using novel anti-β1 monoclonal antibodies. J. Cell Sci. 105:101–111.

60. Pelletier, A. J., S. C. Bodary, and A. D. Levinson. 1992. Signal transduction by the platelet integrin αIIbβ3: induction of calcium oscillations required for protein-tyrosine phosphorylation and ligand-induced spreading of stably transfected cells. Mol. Biol. Cell. 3:989–998.

61. Ramaswamy, H., and M. E. Hemler. 1990. Cloning, primary structure, and properties of a novel human integrin β subunit. EMBO (Eur. Mol. Biol. Organ.) J. 9:1561–1568.

62. Regen, C. M., and A. F. Horwitz. 1992. Dynamics of β1 integrin-mediated adhesive contacts in motile fibroblasts. J. Cell Biol. 119:1347–1359.

63. Repesh, L. A., S. R. Drake, M. C. Warner, S. W. Downing, R. Jrying, E. A. Seftor, and J. B. McCarthy. 1993. Adriamycin-induced inhibition of melanoma cell invasion is correlated with decreases in tumor cell motility and increases in focal contact formation. Clin. & Exp. Metastasis. 11:91–102.

64. Reszka, A. A., Y. Hayashi, and A. F. Horwitz. 1992. Identification of amino acid sequences in the integrin β1 cytoplasmic domain implicated in cytoskeletal association. J. Cell Biol. 117:1321–1330.

65. Richter, J., J. Ng-Sikorski, I. Olsson, and T. Andersson. 1991. Tumor necrosis factor-induced degranulation of adherent human neutrophils is dependent on CD11b/CD18-integrin-triggered oscillations of cytosolic free Ca2+. Proc. Natl. Acad. Sci. USA. 87:9472–9476.

66. Roberts, K., W. M. Yokoyama, P. J. Kehn, and E. M. Shevach. 1991. The vitronectin receptor serves as an accessory molecule for the activation of a subset of γδ T cells. J. Exp. Med. 175:231–240.

67. Schap, D., and P. J. Parker. 1990. Expression, purification, and characterization of protein kinase C-ε. J. Biol. Chem. 265:7301–7307.

68. Schmidt, C. E., A. F. Horwitz, D. A. Lauffenburger, and M. P. Sheetz. 1993.β1 integrin-vitronectin triggers elevation of intracellular free calcium. J. Cell Biol. 120:1003–1010.

69. Solow, J., J. A. Edelman, S. M. Albelda, and C. A. Buck. 1991. Cytoplasmic and transmembrane domains of integrin β1 and β2 subunits are functionally interchangeable. J. Cell Biol. 114:1079–1083.

70. Solow, J., J.-L. Guan, E. E. Marcantonio, J. E. Trevithick, C. A. Buck, and R. O. Hynes. 1989. Expression of normal and mutant avian integrin subunits in rodent cells. J. Cell Biol. 109:853–861.

71. Springer, T. A. 1990. Adhesion receptors of the immune system. Nature (Lond.). 346:425–434.

72. Suzuki, S., Z.-S. Huang, and H. Tanahara. 1990. Cloning of an integrin β1 subunit exhibiting high homology with integrin β3 subunit. Proc. Natl. Acad. Sci. USA. 87:5354–5458.

73. Symington, B. E. 1992. Fibronectin receptor modulates cyclin-dependent kinase activity. J. Biol. Chem. 267:25744–25747.

74. Tada, H., O. Shih, K. Kuroshima, M. Koyama, and K. Tsukamoto. 1986. An improved colorimetric assay for interleukin 2. J. Immunol. Methods. 93:157–165.

75. Takahashi, K., T. Nakamura, H. Adachi, H. Yagita, and K. Okumura. 1991. Antigen-independent T cell activation mediated by a very late activation antigen-like extracellular matrix receptor. Eur. J. Immunol. 21:1559–1562.

76. Tama, W. H.-van Kemenade, E., Y. Van Kooyk, A. J. de Boer, R. J. F. Huijbers, P. Weder, W. van de Kastelee, J. C. M. Metief, and C. G. Figdor. 1992. Adhesion of T and B lymphocytes to extracellular matrix and endothelial cells can be regulated through the β subunit of VLA. J. Cell Biol. 117:461–470.

77. Wagnier, E. A., R. A. Orlando, and D. A. Chershe. 1991. Integrins αvβ3 and αvβ5 contribute to cell attachment to vitronectin but differentially distribute on the cell surface. J. Cell Biol. 113:919–929.

78. Weik, Z., P. M. Treble, O. Behrendtsean, E. Crowley, and C. H. Damsky. 1989. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J. Cell Biol. 109:877–889.

79. Wied, H., J. Buchner, R. Zimmerman, and U. Jakob. 1992. Hsp90 chaperones protein folding in vitro. Nature (Lond.). 358:169–170.

80. Yamasada, A., Y. Nojima, K. Sugita, N. H. Dang, S. F. Schlossman, and C. H. Damsky. 1991. Cross-linking of VLA/CD29 molecule has a co-mitogenic effect with anti-CD3 on CD4 cell activation in serum-free culture system. Eur. J. Immunol. 21:319–325.

81. Yamaguchi, A., K. Tanone, and H. Yaazaki. 1990. Secondary signals required for protein-tyrosine phosphorylation and ligand-induced spreading of stably transfected cells. Mol. Biol. Cell. 3:989–998.

82. Yamada, A., Y. Nojima, K. Sugita, N. H. Dang, S. F. Schlossman, and C. H. Damsky. 1991. Cross-linking of VLA/CD29 molecule has a co-mitogenic effect with anti-CD3 on CD4 cell activation in serum-free culture system. Eur. J. Immunol. 21:319–325.