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# Integrin-dependent Control of Translation: Engagement of Integrin $\alpha_{IIb}\beta_3$ Regulates Synthesis of Proteins in Activated Human Platelets

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**Abstract.** Integrins are widely expressed plasma membrane adhesion molecules that tether cells to matrix proteins and to one another in cell–cell interactions. Integrins also transmit outside-in signals that regulate functional responses of cells, and are known to influence gene expression by regulating transcription. In previous studies we found that platelets, which are naturally occurring anucleate cytoplasts, translate preformed mRNA transcripts when they are activated by outside-in signals. Using strategies that interrupt engagement of integrin  $\alpha_{IIb}\beta_3$  by fibrinogen and platelets deficient in this integrin, we found that  $\alpha_{IIb}\beta_3$  regulates the synthesis of B cell lymphoma 3 (Bcl-3) when platelet aggregation is induced by thrombin. We also found that synthesis of Bcl-3, which occurs via a specialized translation control pathway regulated by mammalian

target of rapamycin (mTOR), is induced when platelets adhere to immobilized fibrinogen in the absence of thrombin and when integrin  $\alpha_{IIb}\beta_3$  is engaged by a conformation-altering antibody against integrin  $\alpha_{IIb}\beta_3$ . Thus, outside-in signals delivered by integrin  $\alpha_{IIb}\beta_3$  are required for translation of Bcl-3 in thrombin-stimulated aggregated platelets and are sufficient to induce translation of this marker protein in the absence of thrombin. Engagement of integrin  $\alpha_2\beta_1$  by collagen also triggered synthesis of Bcl-3. Thus, control of translation may be a general mechanism by which surface adhesion molecules regulate gene expression.

**Key words:** adhesion • integrins • platelets • translation • gene regulation

**I**NTEGRINS are plasma membrane proteins that mediate adhesion of cells to other cells and to matrix structures (Hynes, 1992). Individual  $\alpha$  and  $\beta$  subunits pair to form heterodimers of characteristic ligand specificity that interact via their cytoplasmic domains with cytoskeletal and other intracellular proteins (Hynes, 1992; Shattil and Ginsberg, 1997). Interaction of integrin intracellular domains with cytoplasmic proteins confers the ability to transmit outside-in signals when the extracellular domains are engaged by specific ligands, in addition to providing a mechanism by which affinity and avidity of integrins for their ligands can be regulated (Hynes, 1992; Clark and Brugge, 1995; Schwartz et al., 1995; Shattil and Ginsberg,

1997). Outside-in signaling resulting from integrin engagement triggers a variety of responses in cells, including fluxes in intracellular calcium, sodium–proton exchange and alterations in intracellular pH, phosphatidylinositol metabolism, activation of calpain, focal adhesion kinase, mitogen-activated protein kinases and other enzymes, and induction of nuclear signaling pathways leading to expression of new gene products (Clark and Brugge, 1995; Schwartz et al., 1995; Howe et al., 1998). These biochemical events lead to both rapid and delayed changes in cellular function and phenotype, including motility, growth, and differentiation. Outside-in signaling by integrins is both heterodimer- and cell-specific (Shattil and Ginsberg, 1997) and has been studied frequently in the context of adhesion of cells to matrix ligands. Although integrins are also prototypic tethering factors in cell–cell interactions (Zimmerman et al., 1996), less is known of their outside-in signaling roles in this context compared with cell–matrix interactions.

The mechanisms by which gene expression is regulated

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by cellular adhesion are of considerable interest because this imposes both spatial and biochemical control on the process. Studies of fibroblasts and human monocytic cells demonstrate that engagement of integrins by antibodies against their extracellular domains or by matrix ligands induces activation and/or nuclear translocation of Rel (NF- $\kappa$ B) and other transcription factors, and consequent transcription of specific mRNAs (Schwartz et al., 1995; Juliano, 1996). In some cases, only mRNA transcripts are induced by integrin engagement alone, and a second signal is required for their translation and expression of the corresponding protein (Juliano, 1996). Whether integrins directly or indirectly regulate posttranscriptional pathways is largely unknown, and is a challenging issue to study because of the clear and potent influence of integrin engagement on transcriptional events (Juliano and Haskill, 1993; Schwartz et al., 1995).

Recently, we found that stimulated human platelets synthesize proteins from preformed mRNA in an activation-dependent fashion (Weyrich et al., 1998). Because they are primary nucleate cytoplasts that bear receptors and surface adhesion molecules capable of mediating outside-in signaling (Shattil et al., 1994, 1998), platelets are a unique system in which to study activation-dependent translational events independent of nuclear influences. In activated platelets, the synthesis of several induced proteins is inhibited by the immunosuppressant rapamycin in addition to general inhibitors of translation (Weyrich et al., 1998), indicating the presence of a specialized pathway of translational control regulated by mammalian target of rapamycin (mTOR)<sup>1</sup> (Brown and Schreiber, 1996; Thomas and Hall, 1997). The transcript for B cell lymphoma protein 3 (Bcl-3), an intracellular regulatory factor, is present in platelets and is translated via this pathway when platelets are stimulated with thrombin (Weyrich et al., 1998), making it a useful marker for studies of regulated protein synthesis in this cell type. Here we show that translation of Bcl-3 is an adhesion-dependent event that requires engagement of integrin  $\alpha_{IIb}\beta_3$  in platelets activated by thrombin. Integrin  $\alpha_{IIb}\beta_3$  is expressed only by platelets and megakaryocytes, and transmits outside-in signals in addition to mediating cellular aggregation and adhesion (Phillips et al., 1991; Hynes, 1992; Shattil et al., 1998). We also show that direct engagement of integrin  $\alpha_{IIb}\beta_3$  induces expression of Bcl-3 in the absence of thrombin or other exogenous agonists, and that synthesis of Bcl-3 is induced when platelets adhere to collagen via integrin  $\alpha_2\beta_1$ . These experiments demonstrate for the first time that integrins can directly control expression of gene products at translational checkpoints, and that their influence on the flow of genetic information is not limited to regulation of transcription.

## Materials and Methods

### Cell Isolation

Platelets were isolated using the methods of Hamburger and McEver

1. *Abbreviations used in this paper:* Bcl-3, B cell lymphoma 3; mTOR, mammalian target of rapamycin; PDK1, 3-phosphoinositide-dependent protein kinase 1; PI3K, phosphatidylinositol-3-kinase.

(1990). In brief, human blood was drawn into acid-citrate-dextrose (ACD; 7 ml ACD/42 ml of blood) and was centrifuged (200 g for 20 min) to obtain platelet-rich plasma. Platelet-rich plasma was recentrifuged (500 g for 20 min) in the presence of 100 nM prostaglandin E-1. The supernatant was discarded and platelet pellet was resuspended in 50 ml of Pipes/saline/glucose (5 mM Pipes, 145 mM NaCl, 4 mM KCl, 50  $\mu$ M Na<sub>2</sub>HPO<sub>4</sub>, 1 mM MgCl<sub>2</sub>·6 H<sub>2</sub>O, and 5.5 mM glucose), containing 100 nM of prostaglandin E-1 (Sigma Chemical Co.). The platelet suspension was centrifuged (500 g for 20 min), the supernatant was discarded, and the platelet pellet was resuspended in M199 (phenol red free; Whittaker M.A. Bioproducts). In selected studies, the platelets were suspended in Ca<sup>2+</sup> and Mg<sup>2+</sup>-free HBSS containing 5 mM EGTA to chelate Ca<sup>2+</sup>.  $2.5 \times 10^8$  platelets were used for each experimental point. Platelets were stimulated with thrombin (Sigma Chemical Co.), collagen (from human placenta; Sigma Chemical Co.), fibrinogen (Sigma Chemical Co.), or an activating antibody, D3GP3 (provided by Dr. L.K. Jennings, University of Tennessee, Memphis, TN) for 1 h at 37°C while gently rocking in small volume conical tubes. In selected studies, platelets were preincubated with  $\alpha_{IIb}\beta_3$  blocking antibodies, 7E3 (obtained commercially and provided by Dr. S. Tam, Centocor, Malvern, PA), 10E5 (provided by Dr. B.S. Coller, Mount Sinai School of Medicine, New York), G4120, G4709 (provided by Dr. T. Gadek, Genentech, San Francisco, CA), or integrilin (eptifibatide) (provided by Dr. S. Hollenbach, COR Therapeutics, South San Francisco, CA) before stimulation with thrombin. Isotype-matched antibodies were used as controls as indicated in the text and figure legends ( $\alpha$ -LFA-1 and CD31 from R&D Systems). After 1 h, the platelet pellets were collected and prepared for Western analysis as described below.

Human tissues for examination of platelets in situ were collected at the time of surgical intervention. Surgical tissues were collected according to protocols approved by the University of Utah internal review board after informed consent.

### Platelet Adhesion Assay

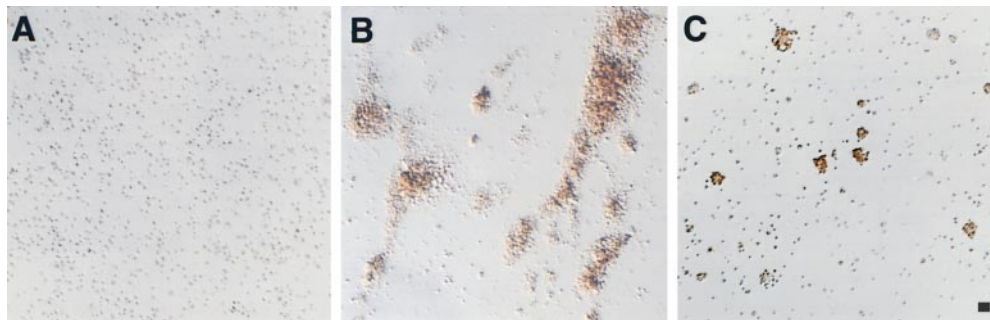
Platelet adhesion to fibrinogen was studied in 4-well polystyrene chambers (Nunc Inc.) precoated overnight at 4°C with HBSS-human serum albumin (2%), which served as the control, fibrinogen (1 mg/ml; Sigma Chemical Co.), or collagen (50  $\mu$ g/ml; Sigma Chemical Co.). Plates were washed three times and blocked for 2 h with 1 ml of HBSS-human serum albumin (2%), and washed three times with HBSS followed by three more washes with HBSS containing 0.01% Tween 20. Residual buffer was removed by aspiration and  $2.5 \times 10^8$  platelets/ml were added to the matrix-coated wells for 1 h at 37°C. After this time, adherent platelets were scraped into Eppendorf tubes and the suspensions were centrifuged for 2 min, 1,000 g at room temperature, and the supernatants were removed. The cell pellets were placed in SDS-PAGE reducing buffer for Western analysis as described below.

### Immunoblotting Procedure

Platelet pellets, collected from activated cells in suspension or those adherent to fibrinogen, were placed in SDS-PAGE reducing buffer, electrophoresed on a 9% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. Western analysis was conducted using affinity-purified, rabbit polyclonal anti-Bcl-3 antibody (Santa Cruz Technology). Immunoreactive protein was detected by affinity-isolated goat anti-rabbit antibody conjugated to peroxidase (Biosource Int.) and an enhanced chemiluminescence detection reagent (Amersham Life Science).

### Immunocytochemical and Immunohistochemical Procedures

Immunocytochemical procedures were performed as described previously, with minor modifications (Weyrich et al., 1996, 1998). In brief, platelets were spun onto glass slides and immediately fixed with 1% paraformaldehyde. After a methanol permeabilization step, the cells were blocked and probed with anti-Bcl-3 (Santa Cruz Technology). Immunoreactive protein for Bcl-3 was detected using an ABC kit from Vectastain (Vector Laboratories, Inc.) for horseradish peroxidase detection that yields a brown immunostain product. Control slides included omission of the primary antibody, omission of the secondary antibody, and/or substitution of nonimmune rabbit IgG. Tissue specimens from abdominal aortic aneurysms were collected and placed in Histochoice MB fixative (Amresco Inc.). After fixation, the specimens were embedded in paraffin, sectioned into 5- $\mu$ m slices, and immunoreactivity for Bcl-3 was assayed as de-



**Figure 1.** Bcl-3 is expressed in aggregated human platelets activated by thrombin. Platelets ( $2.5 \times 10^8/\text{ml}$ ) were isolated as described (Materials and Methods) and treated with control buffer or thrombin (0.1 U/ml) for 1 h at 37°C while being gently rocked in small volume conical tubes. Immunostaining using an antibody against Bcl-3 was done as described

(Materials and Methods; Weyrich et al., 1998). (A) Platelets treated with buffer alone remained single with little or no staining by anti-Bcl-3 when viewed by low power or high power (not shown) microscopy. (B) Large multicellular aggregates formed in response to thrombin with smaller aggregates also visible and few single cells remaining in suspension. The aggregates stained intensely for Bcl-3, obscuring individual cellular detail at low power. (C) Pretreatment with the blocking anti- $\alpha_{\text{IIb}}\beta_3$ , mAb 10E5 (see text), dramatically inhibited platelet aggregation and staining for Bcl-3. As shown, in some experiments inhibition of aggregation was not complete and Bcl-3 protein was present in the scattered small residual aggregates. The figures are representative of multiple experiments with thrombin-stimulated platelets and of six experiments examining the effects of antiintegrin antibodies. Bar, 10  $\mu\text{m}$ .

scribed previously (Weyrich et al., 1993). Sections were viewed and photographed by Nomarski interference contrast optics using a Zeiss AxioPlan light microscope. Tissue collection procedures were approved by the University of Utah Institutional Review Board.

### Aggregometry

0.5-ml aliquots of platelets ( $2.5 \times 10^8/\text{ml}$ ) were preincubated for 5 min at 37°C in the presence of buffer or antibodies before aggregation was initiated by thrombin. Platelets were placed in siliconized cuvettes and aggregation was monitored by a Sienco aggregometer (model DP-247-E) with constant stirring at 1,000 rpm at a constant temperature of 37°C as described previously (Kouns et al., 1990).

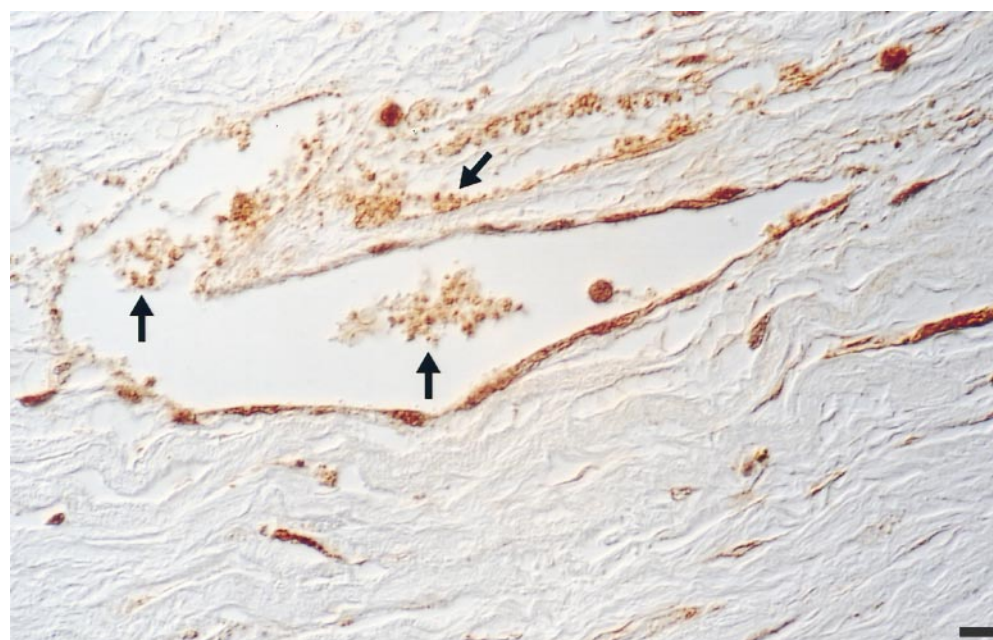
### ELISA

Concentrations of RANTES were measured by ELISA as described previously (Weyrich et al., 1996).

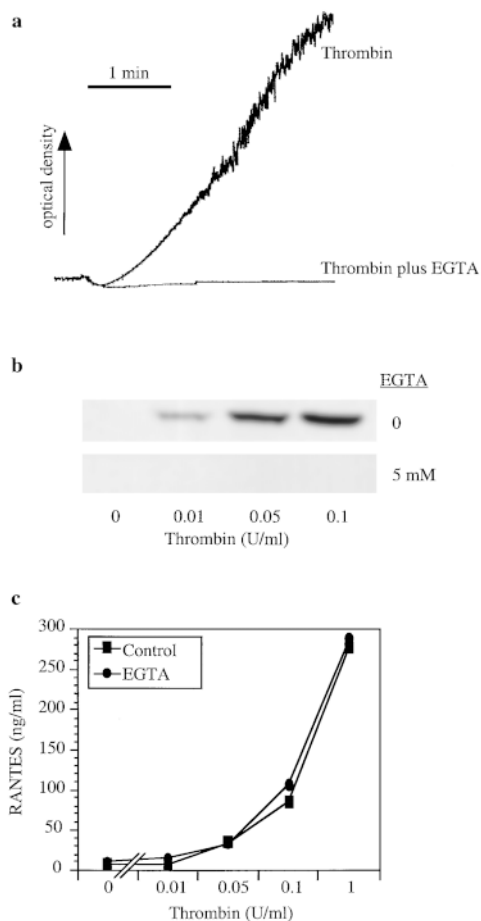
## Results

### *The Expression of Bcl-3 Is Enhanced in Aggregated Human Platelets*

In previous experiments, we found that isolated human platelets translate constitutively present mRNA into proteins in an activation-dependent fashion, that this occurs in platelets stimulated with thrombin, and that Bcl-3 is an informative marker protein to examine in analyses of the synthetic response in this system (Weyrich et al., 1998). In addition, we found that when suspensions of thrombin-stimulated platelets were stained using an antibody against Bcl-3, expression of the protein appeared to be enhanced in aggregated cells compared with single cells. This sug-



**Figure 2.** Bcl-3 is expressed in aggregated human platelets in situ. Tissue sections from vessels resected at the time of surgical intervention in patients with abdominal aortic aneurysms were examined by immunohistochemical analysis. Bcl-3, indicated by the brown reaction product, is present in intravascular platelet aggregates and in platelets adherent to walls of adventitial microvessels (arrows). The adventitia of aneurysmal vessels is an area of inflammatory and thrombotic signaling and cell-cell interactions (Modur et al., 1997). Bcl-3 is also present in endothelial cells and leukocytes in this section, consistent with in vitro observations (Ohno et al., 1990; Pan and McEver, 1995). This figure is representative of surgical specimens from three different subjects. Bar, 10  $\mu\text{m}$ .



**Figure 3.** Removal of extracellular calcium prevents platelet aggregation and inhibits Bcl-3 synthesis. Platelets ( $2.5 \times 10^8/\text{ml}$ ) were isolated as described in Materials and Methods, pretreated for 30 min with vehicle or 5 mM EGTA, and then activated with thrombin for 1 h. (a) Chelation of extracellular calcium abolished platelet aggregation in response to thrombin. (b) In control platelets, thrombin induced Bcl-3 accumulation in a concentration-dependent fashion when assayed by Western analysis or immunostaining (not shown). The accumulation of Bcl-3 was inhibited in platelets stimulated with thrombin in  $\text{Ca}^{2+}$ -free buffer in the presence of the chelator. (c) Thrombin triggered release of RANTES by platelets pretreated with EGTA as well as by control platelets. These results are representative of seven independent experiments.

gested that signaling of protein synthesis in stimulated platelets is influenced by adhesion. To further explore this issue we performed additional immunocytochemical analyses and found that Bcl-3 protein is rapidly expressed in platelet aggregates after thrombin stimulation, with lesser amounts in thrombin-stimulated single cells and little or no protein detectable in platelets in the absence of thrombin (Fig. 1). When the anti-Bcl-3 antibody was deleted or replaced with a control rabbit immunoglobulin, there was no staining of Bcl-3 (Weyrich et al., 1998; data not shown). We also found that Bcl-3 is present in aggregated platelets in microvessels of inflamed tissue (Fig. 2), demonstrating that its synthesis in isolated platelets (Fig. 1) models in vivo events. The accumulation of Bcl-3 in thrombin-stimulated aggregated platelets examined in vitro was time- and

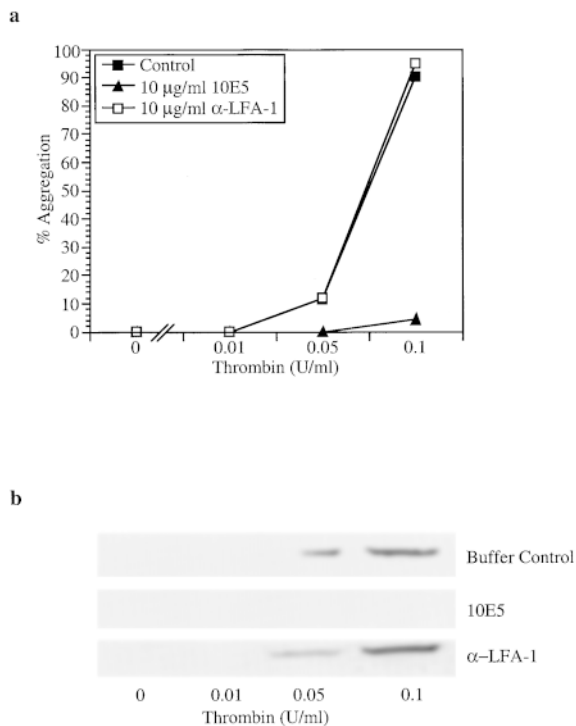
concentration-dependent (Fig. 3 and data not shown), consistent with our earlier studies characterizing its synthesis in this cell type (Weyrich et al., 1998). Because aggregation of human platelets depends on engagement of integrin  $\alpha_{\text{IIb}}\beta_3$  by fibrinogen or von Willebrand factor (Gawaz et al., 1991; Williams et al., 1995), these observations suggested that outside-in signaling via this integrin heterodimer regulates the synthetic pathway leading to expression of the Bcl-3 protein. Therefore, we characterized the role of integrin  $\alpha_{\text{IIb}}\beta_3$  in detail.

### **Chelation of Extracellular Calcium Inhibits Platelet-Platelet Clustering and Abolishes Bcl-3 Synthesis**

We determined if chelation of extracellular calcium interrupts Bcl-3 synthesis, since it is known to block platelet aggregation (Fitzgerald et al., 1985; Shattil et al., 1985). Platelets stimulated with thrombin in calcium-free buffer containing EGTA (5 mM) did not aggregate in response to thrombin (Fig. 3) and did not synthesize Bcl-3 (Fig. 3 b). As a control to test whether signaling via the thrombin receptor was still intact under these conditions, we measured the secretion of RANTES, a preformed chemokine that is stored in alpha granules (Kameyoshi et al., 1992; Weyrich et al., 1996), and found that it was released in response to stimulation (Fig. 3 c). These findings suggested that disruption of adhesive interactions between the aggregating platelets by removal of extracellular cations resulted in impaired synthesis of Bcl-3. Alterations in cation concentrations also have the potential to inhibit intracellular enzymes and other components required for protein synthesis, however, so we pursued this issue further using additional experimental strategies.

### **mAbs Directed Against Integrin $\alpha_{\text{IIb}}\beta_3$ Inhibit Platelet Aggregation and Bcl-3 Synthesis**

We next determined if engagement of the  $\alpha_{\text{IIb}}\beta_3$  integrin is required for Bcl-3 synthesis in thrombin-stimulated platelets. Platelet aggregation occurs when  $\alpha_{\text{IIb}}\beta_3$  heterodimers on adjacent activated platelets are engaged by fibrinogen released from alpha granules or added exogenously (Gawaz et al., 1991; reviewed in Williams et al., 1995). mAb 10E5 blocks binding of fibrinogen to  $\alpha_{\text{IIb}}\beta_3$  and prevents platelet aggregation (Coller et al., 1983; Coller, 1985). Therefore, we determined if mAb 10E5 inhibits Bcl-3 accumulation in stimulated human platelets. As expected, preincubation of platelets with mAb 10E5 markedly attenuated thrombin-induced platelet aggregation compared with control cells in the absence of antibody (Figs. 1 and 4). In addition, Bcl-3 accumulation was inhibited by pretreatment of platelets with mAb 10E5 when examined by immunocytochemistry (Fig. 1) and Western analysis (Fig. 4 b). In some incubations, there were scattered small residual aggregates that were visible by microscopy in suspensions pretreated with mAb 10E5 and then stimulated with thrombin, although the majority of cells did not aggregate; these residual aggregates contained Bcl-3 when examined by immunocytochemical analysis (Fig. 1 c). An isotype-matched mAb against  $\alpha_{\text{L}}\beta_2$  integrin ( $\alpha\text{-LFA-1}$ ; IgG2a) did not inhibit Bcl-3 accumulation in thrombin-stimulated platelets or block platelet aggregation (Fig. 4).



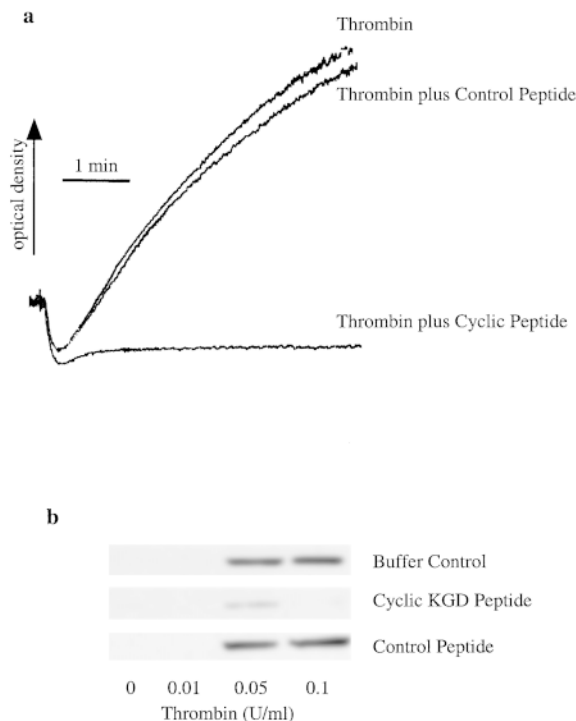
**Figure 4.** An antibody that blocks engagement of integrin  $\alpha_{IIb}\beta_3$  inhibits stimulated synthesis of Bcl-3. Platelets were isolated, preincubated with control buffer, mAb 10E5 (IgG2a), or a control isotype-matched mAb against integrin  $\alpha_L\beta_2$  ( $\alpha$ -LFA-1) for 5 min at 37°C. They were then stimulated with thrombin or treated with control buffer, and platelet aggregation and accumulation of Bcl-3 were examined in parallel as described in Materials and Methods and Figs. 1 and 2. (a) mAb 10E5 inhibits aggregation of thrombin-stimulated platelets whereas anti- $\alpha_L\beta_2$  did not. (b) mAb 10E5 inhibited accumulation of Bcl-3 in platelets stimulated with thrombin in the concentrations shown for 1 h. The isotype-matched control antibody did not attenuate Bcl-3 accumulation compared with that in platelets treated with control buffer alone. The results shown are representative of six independent events.

We performed similar experiments with the Fab fragment of the chimeric human-murine mAb 7E3. This antibody recognizes integrins  $\alpha_{IIb}\beta_3$  and  $\alpha_v\beta_3$ , inhibits fibrinogen binding to platelets, and has been used to block platelet aggregation as a clinical antithrombotic agent (Coller, 1985; Reverter et al., 1996; Coller, 1997). mAb 7E3 attenuated Bcl-3 accumulation in thrombin-stimulated platelets and also inhibited aggregation in parallel incubations (not shown). In contrast, a mAb against  $\alpha_v\beta_3$  did not block Bcl-3 expression.

#### **Peptides That Block Engagement of Integrin $\alpha_{IIb}\beta_3$ Inhibit Synthesis of Bcl-3 in Stimulated Human Platelets**

Binding of fibrinogen to integrin  $\alpha_{IIb}\beta_3$  on activated platelets requires engagement of a dodecapeptide sequence in the  $\gamma$  chain of fibrinogen by the integrin heterodimer, an event that can be blocked by peptides that contain arginine-glycine-asparagine (RGD) sequences (Du et al., 1991; Phillips et al., 1991). Substitution of lysine (K) for arginine

(R) in the RGD sequence confers specificity for integrin  $\alpha_{IIb}\beta_3$  compared with other integrins, and cyclic peptides containing the KGD sequence that are based on the snake venom disintegrin, barbourin, potently inhibit fibrinogen binding to  $\alpha_{IIb}\beta_3$  and platelet aggregation (Scarborough et al., 1991; Scarborough et al., 1993). We first examined linear RGD peptides as antagonists of aggregation-dependent Bcl-3 synthesis in human platelets and found that under some conditions the peptides themselves had weak agonist effect (not shown), consistent with previous observations (Du et al., 1991). Then, we examined a cyclic KGD heptapeptide that specifically binds to integrin  $\alpha_{IIb}\beta_3$  (Schulman et al., 1996; Pursuit Trial Investigators, 1998). The cyclic peptide antagonist inhibited both thrombin-induced platelet aggregation (Fig. 5 a) and Bcl-3 accumulation (Fig. 5 b). The inhibition was concentration-dependent (range 1–10  $\mu$ g/ml) with maximal inhibition at 10  $\mu$ g/ml (Fig. 5 b). A control peptide had no effect. A second blocking cyclic peptide, G4120 (Barker et al., 1992), also inhibited Bcl-3 synthesis in a concentration-dependent fashion, whereas a control peptide did not (not shown). Thus, inhibition of engagement of  $\alpha_{IIb}\beta_3$  integrin with competitive peptides



**Figure 5.** A cyclic KGD peptide that specifically blocks engagement of integrin  $\alpha_{IIb}\beta_3$  inhibits Bcl-3 synthesis in thrombin-stimulated human platelets. Platelets were isolated as described in Materials and Methods, pretreated with a cyclic KGD heptapeptide specific for integrin  $\alpha_{IIb}\beta_3$  or a control peptide (5 min at 37°C), and then activated with thrombin. (a) Thrombin-induced (0.1 U/ml) platelet aggregation was inhibited when platelets were pretreated with the cyclic KGD peptide (10  $\mu$ g/ml) but not by a control peptide in an equivalent concentration. (b) Pretreatment of platelets with the cyclic KGD peptide attenuated Bcl-3 synthesis in thrombin-activated platelets, whereas a control KGD peptide at the same concentration did not. These results are representative of four independent experiments.



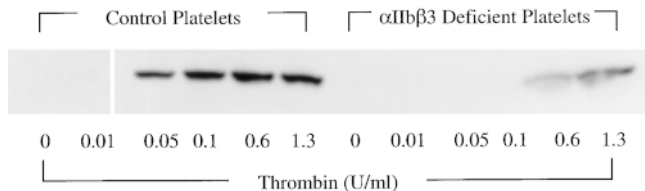
attenuates accumulation of Bcl-3 in platelets stimulated with thrombin (Fig. 5), as does treatment of the platelets with blocking antibodies (Fig. 4).

### ***Bcl-3 Synthesis Is Absent or Reduced When Platelets Deficient in Integrin $\alpha_{IIb}\beta_3$ Are Stimulated with Thrombin***

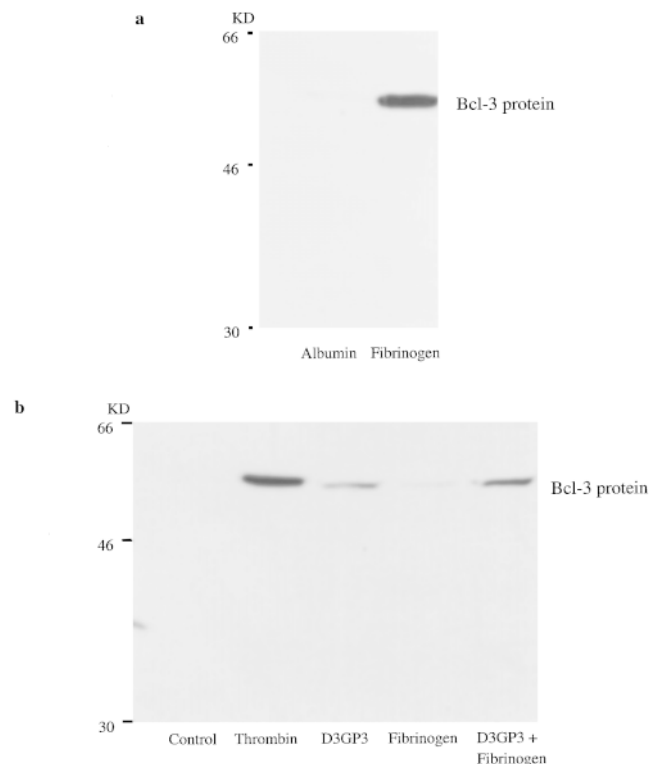
Platelets from patients with Glanzmann thrombasthenia have significant reductions or absence of  $\alpha_{IIb}\beta_3$  on their surfaces (George et al., 1990; Newman and Poncz, 1995). The deficiency in integrin  $\alpha_{IIb}\beta_3$  prevents normal binding of ligands and consequent platelet aggregation, accounting for the hemostatic defect that characterizes these subjects. Platelets from patients with Glanzmann thrombasthenia also have impaired outside-in signaling (Wang et al., 1997). We studied platelets from a patient with type I Glanzmann thrombasthenia that do not aggregate when stimulated by thrombin or a variety of other agonists (Jin et al., 1996). When compared with platelets from a normal control subject isolated in parallel, these mutant platelets exhibited a dramatic defect in accumulation of Bcl-3 in response to thrombin stimulation (Fig. 6). There was no accumulation of Bcl-3 in the integrin  $\alpha_{IIb}\beta_3$ -deficient platelets at concentrations of thrombin (0.05, 0.1 U/ml) that induced synthesis of the protein marker in the simultaneously assayed control platelets (Fig. 6) or in platelets from other control subjects (Figs. 1 and 3–5). At higher concentrations of thrombin, accumulation of Bcl-3 in the  $\alpha_{IIb}\beta_3$ -deficient platelets was attenuated but not absent (Fig. 6).

### ***Direct Activation of Integrin $\alpha_{IIb}\beta_3$ Induces Bcl-3 Synthesis***

We showed previously that adhesion of platelets to purified immobilized fibrinogen induces the synthesis of multiple proteins in the absence of thrombin or another agonist (Weyrich et al., 1998). This is consistent with earlier studies indicating that integrin  $\alpha_{IIb}\beta_3$  on the platelet surface can engage immobilized fibrinogen and transmit outside-in signals without requiring an exogenous agonist, whereas engagement by soluble fibrinogen requires agonist-stimulated cellular activation (Savage and Ruggeri, 1991; Hai-



**Figure 6.** Platelets deficient in integrin  $\alpha_{IIb}\beta_3$  have absent or attenuated synthesis of Bcl-3 when stimulated with thrombin. Platelets ( $2.5 \times 10^8$ /ml) were isolated from a subject with Glanzmann thrombasthenia and from a control subject in parallel and were stimulated with thrombin at the indicated concentrations for 2 h at 37°C. They were then assayed for Bcl-3 by Western analysis as described in Materials and Methods and Fig. 3. Additional measurements indicated equivalent loading of protein in samples of the control and  $\alpha_{IIb}\beta_3$  integrin-deficient platelets (not shown).



**Figure 7.** Engagement of integrin  $\alpha_{IIb}\beta_3$  by immobilized ligand or an activating antibody induces synthesis of Bcl-3 in human platelets. (a) Adhesion of platelets to immobilized fibrinogen induces synthesis of Bcl-3. Isolated platelets were incubated on an immobilized fibrinogen matrix or on immobilized albumin for 1 h at 37°C and then examined for the accumulation of Bcl-3 by Western analysis as described in Materials and Methods and Fig. 3. The fibrinogen matrix and control surface were prepared (Materials and Methods) using a modification of a previously described method (Haimovich et al., 1993). This result is representative of four experiments. (b) An activating antibody against integrin  $\alpha_{IIb}\beta_3$ , D3GP3, triggers synthesis of Bcl-3 in isolated human platelets. Isolated platelets were incubated in suspension with mAb D3GP3 (40  $\mu$ g/ml), with fibrinogen (100  $\mu$ g/ml), or with mAb D3GP3 plus fibrinogen for 1 h at 37°C and then processed for Western analysis for Bcl-3 (Materials and Methods and Fig. 3). In parallel, platelets were treated with thrombin (0.1 U/ml) or control buffer as positive and negative controls, respectively. In contrast to mAb D3GP3 (third and fifth lanes), a control antibody did not trigger Bcl-3 accumulation (not shown). A second experiment yielded similar results.

movich et al., 1993; Shattil et al., 1994). To determine if engagement of integrin  $\alpha_{IIb}\beta_3$  is sufficient to induce translation of the marker protein Bcl-3, we first used this immobilized fibrinogen system. We found that platelets adherent to a fibrinogen matrix accumulated Bcl-3 (Fig. 7 a). In contrast, there was little or no Bcl-3 in platelets incubated in suspension (not shown) or on immobilized albumin in parallel. mAbs 10E5 and 7E3 (see above) inhibited Bcl-3 accumulation in platelets adherent to immobilized fibrinogen. In addition, LY 294002 and Wortmannin, which inhibit phosphatidylinositol-3-kinase (PI3K), blocked Bcl-3 expression in platelets adherent to immobilized fibrinogen (not shown), as they do in thrombin-stimulated aggregated platelets (Weyrich et al., 1998).



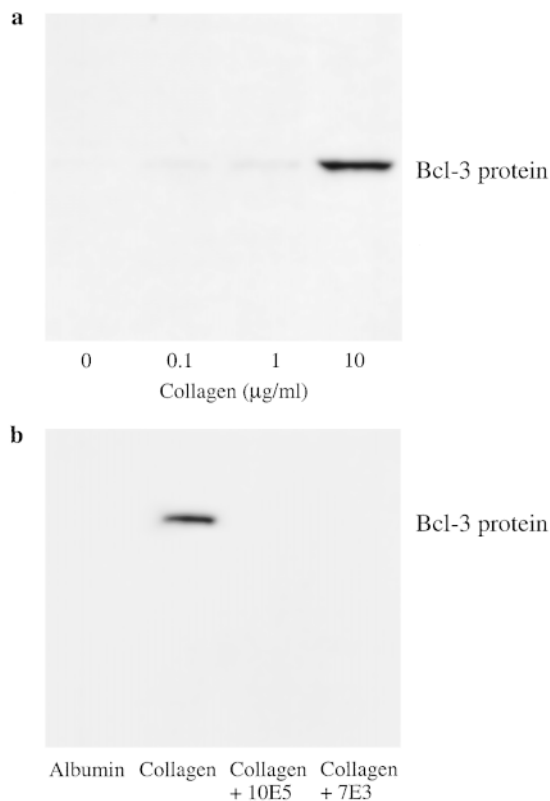
We then used an alternative strategy to ask if engagement of integrin  $\alpha_{IIb}\beta_3$  delivers outside-in signals to the translation pathway that regulates synthesis of Bcl-3. mAb D3GP3 is directed against the  $\beta_3$  chain of the integrin  $\alpha_{IIb}\beta_3$  heterodimer and induces a conformational change that makes the integrin competent to bind fibrinogen and mediate aggregation in the absence of thrombin or another stimulus (Kouns et al., 1990; Kouns and Jennings, 1991). We found that incubation of platelets with mAb D3GP3 resulted in their aggregation (not shown), as previously reported, and also triggered synthesis of Bcl-3 (Fig. 7 b). An isotype-matched antibody against another protein on the platelet plasma membrane, PECAM-1 (CD31), did not induce Bcl-3 synthesis (not shown). When examined by immunocytochemistry, Bcl-3 was predominantly located in aggregated platelets in suspensions treated with D3GP3, with few single platelets showing staining (not shown). The accumulation of Bcl-3 in platelets incubated with mAb D3GP3 was not as great as that in platelets stimulated with thrombin in parallel (Fig. 7 b), consistent with the fact that the antibody induces submaximal aggregation under these conditions (Kouns et al., 1990) (our experiments not shown). Platelets treated with mAb D3GP3 bind exogenously added fibrinogen in an enhanced fashion (Kouns et al., 1990). When we added soluble fibrinogen to the incubation, the accumulation of Bcl-3 was enhanced in platelets incubated with D3GP3 (Fig. 7 b). Exogenous soluble fibrinogen did not induce Bcl-3 synthesis in platelets incubated with the control mAb against PECAM-1 (not shown).

### Collagen Triggers Bcl-3 Synthesis by Human Platelets

Collagen is recognized by integrin  $\alpha_2\beta_1$  as well as by other adhesion molecules on platelets, and treatment of isolated platelets with collagen in solution induces activation of intracellular kinases and aggregation equivalent in magnitude to that triggered by thrombin (Lipfert et al., 1992; Shattil et al., 1994). We found that collagen in solution induced synthesis of Bcl-3 in a concentration-dependent fashion (Fig. 8 a). In addition, we found that platelets adherent to immobilized collagen synthesized Bcl-3 (Fig. 8 b). The accumulation of Bcl-3 in platelets adherent to immobilized collagen was inhibited by a blocking antibody against the integrin  $\alpha_2$  subunit (not shown) and also by mAb 7E3 (see above) (Fig. 8 b) but not by a control mAb against  $\alpha_v\beta_3$  (not shown). The latter findings are consistent with a previous report that mAb 7E3 and an antibody against  $\alpha_2\beta_1$  integrin each inhibited outside-in signaling of platelets when the cells adhered to an immobilized collagen matrix (Haimovich et al., 1993). mAb 10E5 also blocked synthesis of Bcl-3 under similar conditions (Fig. 8 b). Thus, engagement of integrin  $\alpha_2\beta_1$  and/or other surface receptors for collagen together with integrin  $\alpha_{IIb}\beta_3$  may mediate signaling of translational events leading to Bcl-3 synthesis in a costimulatory fashion.

### Discussion

The flow of genetic information is regulated at sequential checkpoints that, together, provide precise control of the expression of protein products (Darnell, 1982; Kozak,



**Figure 8.** Collagen induces synthesis of Bcl-3 in human platelets. (a) Collagen in solution induces Bcl-3 synthesis by human platelets. Platelets were isolated as described in Materials and Methods and incubated in suspension with collagen in the concentrations shown for 1 h at 37°C. Synthesis of Bcl-3 protein was examined by Western analysis. Collagen in solution induced Bcl-3 expression in three additional experiments. (b) Adhesion of platelets to immobilized collagen induces Bcl-3 synthesis. Platelets were pretreated with control buffer, mAb 10E5, or mAb 7E3 as described in Fig. 4 and Materials and Methods and then were incubated on immobilized collagen or on immobilized albumin for 1 h at 37°C. Accumulation of Bcl-3 was examined by Western analysis as described in Materials and Methods. This result is representative of three experiments.

1991). Here we show that integrin  $\alpha_{IIb}\beta_3$  regulates translation of a marker protein, Bcl-3, in thrombin-stimulated aggregated platelets. Integrin  $\alpha_{IIb}\beta_3$  is the principle integrin of human platelets and is a herald member of the integrin family that has yielded many insights into the structure and function of these adhesion proteins (Phillips et al., 1991; Hynes, 1992; Shattil et al., 1994; Shattil et al., 1998). We also found that outside-in signals delivered via integrin  $\alpha_{IIb}\beta_3$  trigger Bcl-3 synthesis when the integrin heterodimer is engaged by immobilized ligand or a function-perturbing antibody. Our findings raise the possibility that a general mechanism by which integrins regulate gene expression is by interacting at posttranscriptional checkpoints, in addition to mediating nuclear signaling and transcriptional events. It has been suggested previously that  $\beta_1$  integrins on leukocytes may influence posttranscriptional steps (Mondal et al., 1995). Also, mechanical signaling via engaged  $\beta_1$  integrins may orchestrate local accumulation of mRNA and ribosomes in the region of focal adhesion

complexes (Chicurel et al., 1998). However, our findings provide the first evidence that outside-in signaling via a specific integrin heterodimer regulates expression of protein products in the absence of nuclear effects.

Human platelets may be a particularly informative system in which to study adhesion-dependent signaling of translation in a primary cell type, and mechanisms elucidated in platelets may also reflect similar processes at the earlier, nucleated, megakaryocyte stage (Weyrich et al., 1998). Platelets have ribosomes and other components required for protein synthesis and carry stable mRNA transcripts (Warshaw et al., 1967; Morgenstern, 1980; Belloc et al., 1982; Kieffer et al., 1987; Newman et al., 1988; Roth et al., 1989; Power et al., 1995). Multiple new proteins are synthesized in thrombin-stimulated platelets and in platelets adherent to immobilized fibrinogen; this synthesis is interrupted by puromycin and cycloheximide and, for a subset of these proteins, by rapamycin (Weyrich, 1998; and our unpublished observations). To date, we have identified Bcl-3 as one of the newly synthesized products and five others as proteins that regulate or are involved in cytoskeletal interactions (Weyrich A.S., N.D. Tolley, M.L. Wade, T.M. McIntyre, S.M. Prescott, Z. Wu, and G.A. Zimmerman, manuscript in preparation). Although mitochondrial transcription occurs in platelets (Agam et al., 1976), Bcl-3 is translated from preformed mRNA and the transcriptional inhibitor actinomycin D does not prevent synthesis (Weyrich et al., 1998). Platelets contain key enzymes in the specialized mTOR pathway that controls translation of a subset of mRNAs with specific structural features (Brown and Schreiber, 1996; Thomas and Hall, 1997), including the mRNA for Bcl-3 (Weyrich et al., 1998). Current evidence from cell lines and transfected cell models indicate that activity in this pathway is initiated by a signal at the plasma membrane followed by a cascade involving PI3K and 3-phosphoinositide-dependent protein kinase 1 (PDK1) and culminating in phosphorylation of the translation repressor eIF4E-binding protein 1 (4EBP-1), causing it to dissociate from eukaryotic translation initiation factor 4E (eIF4E) and allowing cap-dependent translation to proceed (Sonenberg and Gingras, 1998). Phosphorylation and activation of the ribosomal S6 kinase, p70 S6 kinase (p70<sup>S6K</sup>), also occurs. In previous studies of lymphocytic cell lines and fibroblasts, this pathway was shown to be triggered by growth factors and mitogens (reviewed in Brown and Schreiber, 1996; Thomas and Hall, 1997; Peterson and Schreiber, 1998; Sonenberg and Gingras, 1998). In aggregating platelets, PI3K is triggered in an adhesion-dependent fashion and PDK1 is also present (Clark and Brugge, 1995; Banfic et al., 1998a,b). Inhibitors of PI3K block synthesis of Bcl-3 in thrombin-stimulated aggregated platelets (Weyrich et al., 1998) and in platelets adherent to immobilized fibrinogen (this study). In addition, p70<sup>S6K</sup> is present in human platelets (Papkoff et al., 1994) and is activated when they aggregate in response to thrombin (Weyrich, A.S., unpublished experiments). 4EBP-1 is also phosphorylated in thrombin-stimulated platelets and this event and the synthesis of Bcl-3 are blocked by inhibitors of PI3K and by rapamycin (Weyrich et al., 1998). Thus, platelets have critical enzymatic and regulatory molecules that are required for translation control, including components of the mTOR pathway, and the

activities of these systems are influenced by outside-in signals.

Using blocking antibodies, competitive peptides, and deficient platelets from a subject with Glanzmann thrombasthenia, we found that engagement of integrin  $\alpha_{IIb}\beta_3$  regulates Bcl-3 synthesis in aggregating platelets stimulated by thrombin. Thus, signals transmitted by integrin  $\alpha_{IIb}\beta_3$  are linked to translation control pathways. We also found that ligation of integrin  $\alpha_{IIb}\beta_3$  by immobilized fibrinogen or binding of a conformation-altering antibody induces synthesis of Bcl-3 in the absence of thrombin stimulation. These experiments and our previous observations (Weyrich et al., 1998) indicate that engagement of integrin  $\alpha_{IIb}\beta_3$  is sufficient to signal activation of translational pathways and synthesis of a variety of proteins. Binding of fibrinogen to integrin  $\alpha_{IIb}\beta_3$  in the presence of an activating "LIBS" anti- $\beta_3$  antibody triggers PI3K and apparent PDK1 activities, responses that require platelet-platelet contact and aggregation to be maximal (Banfic et al., 1998a,b). Thus, engagement of integrin  $\alpha_{IIb}\beta_3$  can activate key enzymes in the transduction cascade that relay signals from the plasma membrane to translational pathways (see above). Whether there are intracellular signaling cascades that are specific to integrin  $\alpha_{IIb}\beta_3$  (Banfic et al., 1998a) is unknown. In other systems, integrins and growth factors or mitogens use common, rather than unique, intracellular mechanisms to trigger gene expression (Juliano, 1996; Howe et al., 1998). How integrin  $\alpha_{IIb}\beta_3$  interfaces with downstream components of the translation control pathway that regulate phosphorylation of 4EBP1 and p70<sup>S6K</sup> activation is also currently unknown. In a previous study, adhesion of a cell line to immobilized fibronectin, laminin, or vitronectin activated p70<sup>S6K</sup>, implying that regulation of this enzyme is linked to engagement of integrins of both the  $\beta_1$  and  $\beta_3$  classes (Malik and Parsons, 1996). Additional experiments indicated that focal adhesion kinase was partially required for p70<sup>S6K</sup> activation. Whether focal adhesion kinase, which is signaled by integrin  $\alpha_{IIb}\beta_3$  engagement (Lipfert et al., 1992; reviewed in Shattil et al., 1994; reviewed in Clark and Brugge, 1995; Lyman et al., 1997; and reviewed in Shattil et al., 1998), is involved in p70<sup>S6K</sup> activation and translational regulation in platelets remains to be explored.

In cell-cell interactions, signals delivered through adhesion molecules are integrated with signals from surface receptors, such as those for growth factors or chemokines, to yield qualitatively distinct responses (Weyrich et al., 1996; Zimmerman et al., 1996). Signals delivered by integrins and growth factors converge and are integrated in this fashion (reviewed in Schwartz et al., 1995; Juliano, 1996; Sastry and Horwitz, 1996). Our finding that Bcl-3 expression in platelets is induced by thrombin stimulation raises the possibility that outside-in signals transmitted by engagement of integrin  $\alpha_{IIb}\beta_3$  interface and are integrated with those generated by ligation of the thrombin receptor resulting in translation of mRNAs. The thrombin receptor and integrin  $\alpha_{IIb}\beta_3$  transmit convergent signals to other response pathways in human platelets (Ferrell and Martin, 1989; Golden et al., 1990; Clark et al., 1994; Shattil et al., 1994; Cichowski et al., 1996). In our experiments, expression of Bcl-3 in thrombin-activated platelets required engagement of integrin  $\alpha_{IIb}\beta_3$  at concentrations of thrombin

(0.01–0.1 U/ml) that triggered maximal or near-maximal platelet aggregation, and was blocked by inhibitory antibodies or peptides against  $\alpha_{IIb}\beta_3$  (see Results). The results argue that at these concentrations ligation of the thrombin receptor is not sufficient to induce translation. At higher concentrations of thrombin, there appeared to be an  $\alpha_{IIb}\beta_3$ -independent mechanism of signaling when platelets from a subject with Glanzmann thrombasthenia were studied (Fig. 6). This is potentially due to differential signaling through other receptors on platelets that recognize thrombin (Schmidt et al., 1998). Alternatively, this may represent the activity of a small number of residual copies of integrin  $\alpha_{IIb}\beta_3$  on the platelets that we used for this study (Jin et al., 1996).

In addition to integrating of signals delivered via receptors for mitogens and growth factors, integrins of different classes may also signal cooperatively (Schwartz et al., 1995; Juliano, 1996). Our experiments in which adhesion of platelets to immobilized collagen induced synthesis of Bcl-3 (Results and Fig. 8) indicate that integrin  $\alpha_{IIb}\beta_3$  and integrin  $\alpha_2\beta_1$  cooperatively signal translation events. We found that an antibody against the  $\alpha_2$  subunit of  $\alpha_2\beta_1$  integrin, which recognizes collagen, and mAb 7E3 and 10E5, which block ligand binding by integrin  $\alpha_{IIb}\beta_3$ , each inhibited Bcl-3 synthesis in platelets that adhered to immobilized collagen matrices (Results). One explanation for this experimental outcome is that adhesion of individual platelets to immobilized collagen caused outside-in signaling via integrin  $\alpha_2\beta_1$  and triggered degranulation and local secretion of fibrinogen, with secondary formation of microaggregates caused by binding of fibrinogen to integrin  $\alpha_{IIb}\beta_3$  on adjacent platelets. Haimovich et al. (1993) reported that microaggregate formation occurs when platelet suspensions are incubated on immobilized collagen and that this is blocked by mAb 7E3. Thus, it is possible that engagement of integrin  $\alpha_{IIb}\beta_3$  by endogenously released fibrinogen alone signals expression of Bcl-3 under these conditions. Alternatively, engagement of integrin  $\alpha_2\beta_1$  may signal directly to the mTOR pathway when platelets bind to immobilized collagen and additionally triggers local secretion of fibrinogen and engagement of integrin  $\alpha_{IIb}\beta_3$ , concomitantly inducing translation of Bcl-3. Although these two possibilities cannot be resolved yet, our results are consistent with cooperative interaction of the two platelet integrins in regulating translational events. Additional evidence for cooperative interaction between integrins  $\alpha_{IIb}\beta_3$  and  $\alpha_2\beta_1$  has also been reported (Coller et al., 1989; Lipfert et al., 1992; Savage et al., 1998). Whether other receptors for collagen (Shattil et al., 1994; Savage et al., 1998; Watson and Gibbins, 1998) also signal translation in adherent platelets is unknown at this time.

Our finding that engagement of integrins regulates synthesis of proteins in platelets (Results and Weyrich et al., 1998) suggests that control of translation is a general mechanism by which integrins and other classes of adhesion molecules influence gene expression. Adhesion-dependent signaling, a process by which integrins and other adhesion molecules can specifically modulate or induce synthesis of particular gene products, adds spatial regulation to this process (Juliano, 1996; Schwartz et al., 1996; Zimmerman et al., 1996). In addition to spatial regulation, activation of translation pathways by outside-in signals de-

livered through integrins or other adhesion molecules can rapidly induce synthesis of proteins from preformed mRNA, influencing the temporal sequence of expression of gene products. Specific modulation by integrins of translation checkpoints, which are downstream of transcription, mRNA processing, nuclear export, mRNA degradation, also adds precision and variety in signaling of gene expression that would not be available if transcription were the only point of influence (Darnell, 1982). Our ongoing studies indicate that translation control occurs when other adhesion molecules besides integrins are engaged (Mahoney, T.S., A.S. Weyrich, G.A. Zimmerman, T.M. McIntyre, S.M. Prescott, manuscript in preparation), suggesting that signaling to translational control pathways is a general mechanism of adhesion-dependent regulation of gene expression.

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