Integrins Regulate the Intracellular Distribution of Eukaryotic Initiation Factor 4E in Platelets

A CHECKPOINT FOR TRANSLATIONAL CONTROL*

Recent evidence from our laboratory demonstrates that platelets synthesize numerous proteins in a signal-dependent fashion (Pablo, R., Weyrich, A. S., Dixon, D. A., Bray, P. F., McIntyre, T. M., Prescott, S. M., and Zimmerman, G. A. (1999) J. Cell Biol. 144, 175–184; Weyrich, A. S., Dixon, D. A., Pablo, R., Elstad, M. R., McIntyre, T. M., Prescott, S. M., and Zimmerman, G. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5556–5561). Protein synthesis in platelets is controlled at the translational level; however, the mechanisms of regulation are not known. Here we demonstrate that translation initiation factors are redistributed to mRNA-rich areas in aggregated platelets, an event that induces protein synthesis. Interrogation of cDNA arrays revealed that platelet-derived mRNAs are primarily associated with the cytoskeletal core. In contrast, eukaryotic initiation factor 4E (eIF4E), the essential mRNA cap-binding protein that controls global translation rates, is localized in the membrane skeleton and soluble fraction of platelets, physically separated from most mRNAs. Platelet activation redistributes eIF4E to the cytoskeleton and increases interactions of eIF4E with mRNA cap structures. Redistribution of eIF4E to the mRNA-rich cytoskeleton coincides with a marked increase in protein synthesis, a process that is blocked when intracellular actin is disrupted. Additional studies demonstrated that β3 integrins are the primary membrane receptor that distributes eIF4E within the cell. These results imply that integrins link receptor-mediated pathways with mRNA-rich cytoskeletal domains and thereby modulate the organization of intracellular translational complexes. They also indicate that the functional status of eIF4E is regulated by its intracellular distribution.

Although platelets retain numerous mRNAs from megakaryocytes, these mRNAs are generally not translated in quiescent cells (1–4). However, we recently demonstrated that cellular activation globally increases protein synthesis indicating that the translational apparatus is intact and functional in platelets (2). Regulated protein synthesis requires aggregation mediated by αIIBβ3 (1), the major integrin that controls intracellular adhesion among platelets (5). Because platelets are anucleate cytoplasts, our results indicate that αIIBβ3 integrins modulate protein synthesis at the translational level (1). Other studies have implicated integrins as well, demonstrating that adhesion to extracellular matrix induces the rapid translation of preexisting mRNAs in nucleated cells (6, 7). These results suggest that outside-in signals delivered by integrins, in addition to regulating a host of other postligand binding events, modulates protein synthesis at the translational level.

Although integrins regulate translation, the mechanisms by which they do so are not known. Translation rates are primarily controlled at three steps: initiation, elongation, and termination. Regulation at the initiation phase is considered predominant and a primary target for translational control because it recruits ribosomes to mRNA (8). Eukaryotic initiation factor 4E (eIF4E) executes the pivotal function of bridging virtually all mRNAs to ribosomes (8). In nucleated cells, eIF4E activity is controlled by its transcription, phosphorylation, and binding to 4E-BP (eIF4E-Binding Protein) repressor proteins (9). Cellular stress, cytokines, and growth factors evoke intracellular signaling pathways that influence eIF4E activity (9). Here we demonstrate that outside-in signals delivered by integrins also regulate the functional status of eIF4E in platelets. However, instead of directly influencing eIF4E activity, αIIBβ3 integrins target this initiation factor to subcellular cytoskeletal domains that contain numerous mRNAs. Redistribution of eIF4E is required for signal-dependent protein synthesis, providing evidence that intracellular spatial localization is an important determinant of eIF4E function.

EXPERIMENTAL PROCEDURES

Isolation and Activation of Platelets in Suspension—Washed human platelets were isolated according to protocols previously published by our laboratory (2, 10). Platelets were activated with thrombin, the thrombin receptor activating peptide (TRAP), platelet-activating factor (PAF), or arachidonic acid (AA) (Calbiochem). In selected studies, the cells were pretreated with αIIBβ3 antagonists (Integrin or monoclonal antibody cTE3), rapamycin, or p38 mitogen-activated protein kinase inhibitors (Calbiochem).

cDNA Arrays—Total RNA was extracted from the cytoskeletal core, membrane skeleton, and soluble fractions of platelets (5 × 105 platelets) (2, 11). Five µg of RNA was converted to 32P-labeled first strand cDNA using Superscript Reverse Transcriptase (Life Technologies, Inc.) and a primer mix obtained from CLONTECH (Palo Alto, CA). The labeled probe mix was subsequently hybridized to atlas array number 7742-1 (CLONTECH) as described by the manufacturer.

Isolation of Platelet Cytoskeletal Compartments and Protein Analysis—Detergent separation of platelet cytoskeletal compartments was conducted according to previous methods (11). Proteins were examined...
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**RESULTS**

**mRNAs Are Associated with the Cytoskeletal Core**—The platelet cytoskeleton contains two actin filament-based components: the cytoskeletal core and the membrane skeleton that coats the cell periphery (14). Initially, we determined if mRNAs in unstimulated platelets are partitioned to these cytoskeletal domains. Resting platelets were lysed in Triton-X containing buffer, and the cytoskeletal core, which contains cross-linked actin filaments and other cytoskeletal elements (15), the membrane skeleton, and the soluble fraction were isolated. A screen of a cDNA array demonstrates that platelets contain numerous mRNAs and the majority of these transcripts are associated with the cytoskeletal core of resting platelets (Fig. 1, left panel). A small number of transcripts are found in the soluble fraction of unstimulated platelets (Fig. 1, right panel). We did not detect mRNAs in the membrane skeleton (data not shown). Thrombin slightly increased the number of mRNAs associated with the cytoskeletal core (data not shown). This increase was a consequence of mRNAs being transferred from the soluble fraction of resting platelets to the cytoskeletal core of stimulated platelets (data not shown).

**eIF4E Is Redistribution to the Cytoskeletal Core of Activated Platelets**—We next examined the topography of eIF4E in resting and activated platelets. We found that platelets contained eIF4E, consistent with a recent report (16). Further analysis demonstrated that eIF4E was confined to the membrane skeleton and soluble fraction of resting platelets (Fig. 2). Actin, β3 integrins, and vinculin were found in the same regions, consistent with previous studies (11). None of these proteins, with the exception of actin, were found in the cytoskeletal core of resting cells. Thrombin stimulation rapidly redistributed eIF4E, β3 integrins, and vinculin to the cytoskeleton (Fig. 2).

We subsequently immunolocalized eIF4E in platelets to confirm that its intracellular distribution changes in response to cellular activation. In these studies, phalloidin was used to distinguish cross-linked actin filaments, a primary component of the cytoskeletal core (17). In resting platelets, phalloidin diffusely stained actin and did not co-localize with eIF4E (Figs. 3, A–C). Following thrombin stimulation, the platelets aggregated to one another and eIF4E co-localized with actin in cell contact areas (Figs. 3, D–F).

**Platelet Activation Increases eIF4E Binding to a mRNA Cap Homologue**—Our results demonstrate that eIF4E is physically separated from mRNAs in resting platelets but is redistributed to mRNA-rich cytoskeletal domains in response to platelet activation. We subsequently used an in vitro mRNA cap binding assay to determine whether platelet-derived eIF4E was capable of interacting with a methylated mRNA cap homologue in activated cells (13). In resting platelets, only a small amount of eIF4E bound to the cap homologue, and thrombin markedly enhanced cap binding (Fig. 4A). eIF4E extracted from thrombin-activated platelets did not interact with non-methylated beads demonstrating the specificity of eIF4E for the methylated mRNA cap homologue (Fig. 4A, last lane). Other platelet agonists, including TRAP, AA, and PAF all increased binding of eIF4E to the methylated mRNA cap homologue (Fig. 4B and data not shown).

**Interruption of eIF4E Redistribution Blocks Signal-dependent Protein Synthesis**—We then determined if redistribution of eIF4E to mRNA-rich cytoskeletal domains is required for protein synthesis. In these studies, we pretreated platelets with cytochalasin D, which blocks agonist-induced polymerization of actin filaments and intracellular motile events (17). We found that cytochalasin D prevented thrombin-induced redistribution of eIF4E to the cytoskeletal core (Fig. 5A). We then examined Bcl-3 and IL-1β protein synthesis because mRNAs for these gene products are translated by activated platelets (2, 25). We first confirmed that mRNA for Bcl-3 and IL-1β is present in the cytoskeletal core of resting platelets (Fig. 5B). We subsequently examined Bcl-3 and IL-1β protein synthesis and found that resting platelets did not contain either protein but synthesized

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1 The abbreviations used are: IL, interleukin; PCR, polymerase chain reaction; eIFs, eukaryotic initiation factors.
these gene products following activation (Fig. 5C). Synthesis of Bcl-3 is required for platelet-dependent retraction of the fibrin network2, and synthesis and processing of IL-1β/H9252 by platelets, at concentrations reported here (Fig. 5C), increases the adhesive-ness of endothelial cells for neutrophils (25). Cytochalasin D decreased synthesis of Bcl-3 and IL-1β/H9252 protein (Fig. 5C). Similarly, signal-dependent global protein synthesis, as measured by incorporation of [35S]methionine into intracellular and se-creted proteins, was markedly attenuated by cytochalasin D (Fig. 5D).

**Fig. 4.** Platelet activation increases eIF4E binding to an mRNA cap homologue. Platelets were left quiescent (C) or stimulated for the indicated time points. After each time point, the cells were lysed and incubated with m’GTP-Sepharose beads as described previously (13). The top panel represents a typical experiment with thrombin (0.1 units/ml) used as an agonist. The lane at the far right illustrates the results using lysates from thrombin-activated platelets (5 min) that were incubated with non-methylated Sepharose beads in place of m’GTP-Sepharose beads. The same experimental procedures were repeated in the bottom panels except that TRAP (25 μM) and arachidonic acid (160 μM) were used as agonists instead of thrombin. This figure is representative of five independent experiments.

**Fig. 5.** Disruption of eIF4E trafficking inhibits protein synthesis. A, platelets were pretreated with 1 μM of cytochalasin D (Cyt) and stimulated with 0.1 units/ml of thrombin for the designated time peri-ods. Proteins from the cytoskeletal core and membrane skeleton were separated by SDS-polyacrylamide gel electrophoresis and immuno-botted for eIF4E and β3 integrins. B represents PCR analysis of Bcl-3 and IL-1β mRNa isolated from the cytoskeletal core (Cc) and soluble (S) fractions of resting (C) and activated (Thr) platelets (0.1 units/ml thrombin for 15 min). C depicts Bcl-3 (Western analysis) and IL-1β (enzyme-linked immunosorbent assay) protein in resting, thrombin-stimulated, and thrombin-stimulated platelets that were pretreated with cytochalasin D (1 μM). For analysis of Bcl-3, the platelets were activated with either 0.01 or 0.05 units/ml thrombin for 1 h (2). For analysis of IL-1β, the platelets were activated with 0.01 or 0.05 units/ml thrombin in the presence of 100 μg/ml fibrinogen for 8 h (25). The asterisk designates p < 0.05 compared with platelets stimulated with thrombin. D depicts a representative autoradiogram of [35S]methionine incorpo-ration (8 h) into intracellular and secreted proteins of unactivated platelets (C), thrombin-activated platelets (Thr), and thrombin-acti-vated platelets pretreated with cytochalasin D (Cyt). A–D are repre-sentative of three independent experiments. The bars in C are the mean ± S.E.

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2 A. S. Weyrich, S. Lindemann, N. D. Tolley, D. A. Dixon, M. L. Wade, T. M. McIntyre, S. M. Prescott, and G. A. Zimmerman, unpublished observations.
Integrins Regulate eIF4E Distribution in Platelets

Translation of mRNA is regulated at multiple checkpoints that allow the cell to precisely control protein synthetic patterns. A prominent level of regulation occurs at the initiation step where eukaryotic initiation factors (eIFs) catalyze translation. eIF4E is the major rate-limiting initiation factor because it docks the eIF4F complex on the cap structure of the 5'-terminus of virtually all mRNAs. Thus, eIF4E regulates global translation rates in eukaryotic cells. Here, we show that mRNAs are associated with the cytoskeletal core of resting platelets, physically separated from eIF4E. Platelet activation redistributes eIF4E to the mRNA-rich cytoskeleton, a process that is required for signal-dependent protein synthesis. Integrins regulate translational events. Integrin binding can induce the movement of mRNA and ribosomes to focal adhesion complexes that contain specific cytoskeletal components (18). Gene expression can also be controlled locally by targeting mRNAs to specialized cytoskeletal domains (19).

We previously demonstrated that platelets synthesize proteins in a signal-dependent fashion (2). Because platelets are anucleate cells, these results demonstrated that signals transmitted by integrins are linked to translational control pathways. However, little is known about the exact mechanisms by which integrins regulate translational events. Integrin binding can induce the movement of mRNA and ribosomes to focal adhesion complexes that contain specific cytoskeletal components (18). Gene expression can also be controlled locally by targeting mRNAs to specialized cytoskeletal domains (19). In resting platelets, we found that the majority of mRNAs are associated with the cytoskeletal core, physically separated from eIF4E.

FIG. 6. Integrins control eIF4E distribution in platelets. A, this figure depicts a typical aggregation tracing of platelets treated with or without 10 μg/ml Integrin (Anti-αIIbβ3). B, platelets were pretreated with (Anti-αIIbβ3) or without Integrin (10 μg/ml) and stimulated with thrombin for the designated times. At the end of each time point, eIF4E protein levels were determined in the cytoskeletal core and membrane skeleton as described under “Experimental Procedures.” C, platelets pretreated with (Anti-αIIbβ3) or without Integrin (C) were prepared for eIF4E cap binding studies as described under “Experimental Procedures.” Fig. 6 is representative of three independent experiments.

link this structure to the underlying cytoskeleton (11, 14), we postulated that β3 integrins target eIF4E to mRNA-rich cytoskeletal domains in response to platelet activation. When integrin αIIbβ3-dependent aggregation was blocked (Fig. 6A), eIF4E did not associate with the cytoskeleton of thrombin-stimulated platelets (Fig. 6B). In addition, inhibition of αIIbβ3-dependent aggregation markedly reduced eIF4E binding to the mRNA cap homologue (Fig. 6C).

Interruption of αIIbβ3-dependent Aggregation Does Not Inhibit Phosphorylation of eIF4E or 4E-BP1—We next determined if integrins also regulate eIF4E function through phosphorylation of eIF4E or its repressor protein, 4E-BP1. We found that eIF4E and 4E-BP1 are phosphorylated in a signal-dependent fashion in platelets (Fig. 7A). Phosphorylation of eIF4E and 4E-BP1 was inhibited by SB203580, a mitogen-activated protein kinase inhibitor, and rapamycin, respectively (Fig. 7A and B). However, inhibition of αIIbβ3-dependent aggregation did not block eIF4E or 4E-BP1 phosphorylation (Fig. 7A and B, respectively) indicating that integrins do not directly influence these responses.

DISCUSSION

Translation of mRNA is regulated at multiple checkpoints that allow the cell to precisely control protein synthetic patterns. A prominent level of regulation occurs at the initiation
Segregation of the translational machinery in resting platelets provides an explanation for limited constitutive protein synthesis in these cells, as reported by numerous investigators (2, 20, 21). αIIbβ3-dependent aggregation redistributes β3 integrins and eIF4E to the mRNA-rich cytoskeleton. When cytochalasin D or αIIbβ3 antagonists disrupt intracellular trafficking, signal-dependent translation of IL-1β, Bcl-3, and other mRNAs is inhibited (see Fig. 5 and Refs. 1 and 25). Presumably, this is due to the inability of eIF4E to interact with mRNAs associated with the cytoskeletal core, a hypothesis that is supported by our data demonstrating that inhibition of αIIbβ3-dependent aggregation prevents eIF4E from interacting with a specific mRNA cap homologue. The association of mRNAs with the cytoskeletal core also suggests that the cytoskeleton serves as a scaffold for regulated protein synthesis by platelets. In other cell types, the major sites of translation include the endoplasmic reticulum and the cytoskeleton (22). Because mature platelets generally do not contain endoplasmic reticulum (23, 24), the cytoskeletal network, which is extensive in platelets, is a logical site for regulated translation.

Although not previously considered, our data demonstrate that spatial targeting of eIF4E within cells is an important mechanism that controls eIF4E function. In resting platelets, eIF4E is topographically found in the soluble fraction and the membrane skeleton. The membrane skeleton is a lattice-like structure that coats the cytoplasmic face of the plasma membrane. It is composed of short actin filaments, actin-binding proteins, spectrin, and vinculin (17). It also contains GP Ib-IX, αGPIa-IIa, and αIIbβ3 (17). Association with the membrane skeleton allows these adhesion molecules to play an important role in binding signaling molecules at sites of integrin-cytoskeletal junctions and thereby mediate signal transduction events in platelets. It is also believed that αIIbβ3 regulates the distribution of membrane skeletal components within the cell (11). This notion is supported by studies where platelets from Glanzmann thrombasthenic patients do not efficaciously redistribute proteins to the cytoskeletal core in response to activation (11). The finding that eIF4E is found in the membrane skeleton suggests that it is ideally positioned for communication with transmembrane spanning proteins including αIIbβ3 and other cell surface receptors. The proximity of eIF4E to membrane receptors may also facilitate eIF4E phosphorylation in platelets, which occurs within 1 min of poststimulation.

Tight regulation of eIF4E in platelets is not surprising since it is considered a primary target for translational control in nucleated cells (8). Extracellular stimuli, such as hormones, growth factors, and mitogens augment translation rates by increasing the transcription of eIF4E or the phosphorylation of eIF4E and its repressor protein, 4E-BP1. In platelets, p38 mitogen-activated protein kinase and mTOR-dependent pathways inhibit eIF4E and 4E-BP1 phosphorylation, respectively. Under the conditions of these studies, interruption of αIIbβ3-dependent aggregation does not alter thrombin-induced phosphorylation of these proteins indicating that signal-dependent protein synthesis in platelets is controlled at multiple checkpoints.

Our data provide new evidence that αIIbβ3 integrins control the intracellular distribution of eIF4E and thereby regulate its function in platelets. Inhibition of platelet aggregation and subsequent protein synthesis (1, 25) by αIIbβ3 antagonists may partially explain their clinical efficacy in reducing the severity of arterial restenosis and associated cardiovascular events. Our results also indicate that in addition to transcription and phosphorylation, the functional status of eIF4E is regulated by its intracellular distribution. Spatial targeting of eIF4E to mRNA-rich areas may control translation rates in nucleated cells as well.

Acknowledgments—We are grateful to Guy A. Zimmerman and other colleagues at the Human Molecular Biology and Genetics Program for their helpful comments and critical reading of the manuscript.

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