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Pak regulates calpain-dependent degradation of E3b1

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Abstract

E3b1, a binding partner of Eps8, plays a critical role in receptor tyrosine kinase (RTK)-mediated Rac activation by facilitating the interaction of Eps8 with Sos-1 and the consequent activation of the Rac-specific guanine nucleotide exchange factor activity of Sos-1. Here we present evidence that E3b1 levels are regulated by the Ca^{2+}-activated protease calpain, and also by Pak, a downstream target of Rac signaling. Serum starvation of Rat2 or COS7 cells resulted in rapid loss of E3b1 that was reversed by calpain inhibitors. Loss was also prevented by expressing the constitutively active Pak1 mutant, $Pak1^{H83,86L}$. Activation of endogenous Pak by platelet-derived growth factor or the constitutively active Rac1 mutant, $Rac1^{G12V}$, also inhibited degradation. In contrast, inhibition of endogenous Pak activity by expressing the Pak auto-inhibitory domain caused degradation of over-expressed E3b1 even in the presence of serum. Taken together, these findings indicate that E3b1 is down-regulated by calpain activation and stabilized by Pak activation. They also suggest that RTK-mediated Rac activation can be modulated by changes in the level of E3b1 in response to signals that affect the activity of calpain or Pak.

Keywords: E3b1; Calpain; Pak; Rac; Serum starvation

Signaling mediated by a receptor tyrosine kinase (RTK) requires sequential activation of the small GTPases Ras and Rac [1]. Son of sevenless (Sos-1), a bifunctional guanine nucleotide exchange factor (GEF) for Ras and Rac, plays a pivotal role in this sequential activation of the GTPases by switching its activity from Ras-GEF to Rac-GEF [2,3]. The GEF specificity of Sos-1 is determined by the various molecular complexes in which it is engaged. The interaction between Sos-1 and Grb2 that is induced by RTK activation stimulates GEF activity for Ras and leads to the well-characterized Ras signaling [4,5]. In contrast, Sos-1 in a tricomplex with E3b1 and Eps8 exhibits GEF activity for Rac and activates Rac signaling. Although the detailed mechanism that influences the formation of the different molecular complexes is not fully understood, E3b1 has a critical role in the process [1–3]. E3b1 (also known as Abl interactor protein 1, Abi-1) was originally identified as a binding partner of c-Abl [6] and also of Eps8 [7]. E3b1 contains PEST motifs, proline-rich regions, and a Src homology 3 (SH3) domain. It binds to the SH3 domain of Eps8 through its proline-rich region [7], and also to Sos-1 through its SH3 domain [8]. Thus, E3b1 acts as a scaffold protein supporting Sos-1 and Eps8. Because Grb2 and E3b1 bind through their SH3 domains to the same binding site on Sos-1, an increase in the level of E3b1 reduces Sos-1 binding to Grb2 and decreases Ras activation while activating Rac [3]. Thus, by facilitating the formation of the Sos-1–E3b1–Eps8 tricomplex, E3b1 helps to propagate signals because the upstream activation of Ras is rapidly switched off, while Rac is activated downstream. On the other hand, disruption of the tricomplex by removing Eps8 by mutation or by expressing an E3b1 mutant that cannot interact with Eps8 impairs Rac activation and Rac-dependent membrane ruffling [3]. It has been suggested that Ras effectors or Ras-independent pathways are involved in the activation of Rac by modulating the Sos-1–E3b1–Eps8 tricomplex and Rac-GEF [3].

Competition of E3b1 with Grb2 for the same binding site on Sos-1 as well as the scaffolding role of E3b1 in the formation of the tricomplex suggests that modulation of the E3b1 protein level could regulate RTK-mediated

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Rac signaling. Indeed, depletion of E3b1 by microinjecting antibodies into fibroblasts blocked Rac-dependent membrane ruffling in response to platelet-derived growth factor (PDGF) [1]. Moreover, expression of oncogenic forms of the Abl and Src tyrosine kinases triggered degradation of Abi-2 protein (which is closely related to E3b1/Abi-1) by the ubiquitin-dependent 26S proteasome pathway [9,10]. Down-regulation of Abi-2 occurs via a Ras-independent pathway and may contribute to the development of Bcr-Abl-positive leukemias.

The E3b1 amino acid sequence contains multiple PEST motifs. These are peptide regions enriched in proline (P), glutamic acid (E), aspartic acid (D), serine (S), and threonine (T), that are thought to be signals for intracellular degradation by the ubiquitin-dependent 26S proteasome pathway, or by calpain [11,12]. While a considerable body of evidence supports the idea that PEST motifs target proteins for degradation by the 26S proteasome [13], the role of calpain in the degradation of PEST-containing proteins is controversial [11,14]. As described above, down-regulation of Abi-2 by ubiquitin-dependent degradation has been reported. However, the possible role of calpain in modulating E3b1 levels has not been explored.

Here we present evidence that upon serum starvation E3b1 is destroyed by calpain-dependent degradation. Degradation was blocked by activating its own downstream effectors, Rac and Pak, and contributed to stabilizing its level. Modulation of E3b1 by these mutually antagonistic mechanisms may provide a flexible means of regulating RTK-mediated Rac signaling.

Materials and methods

Cell culture, transfection, and PDGF treatment. Rat2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 0.1 mM non-essential amino acids (Life Technologies), and penicillin–streptomycin (Life Technologies) in 5% CO_2 at 37 °C. Calpain inhibitor I (ALLN), calpain inhibitor II (ALLM), E-64, calpeptin, and lactacystin were purchased from Calbiochem. COS7 cells and NIH3T3 cells were maintained in DMEM supplemented with 10% FBS and penicillin–streptomycin in 10% CO_2 at 37 °C. Transient transfection of cells with mammalian expression vectors was performed with LipofectAMINE^{PLUS} (Invitrogen) according to the manufacturer's instructions. For PDGF treatment, NIH3T3 cells were serum-starved for 10 min by incubation in DMEM with or without PDGF-BB (Life Technologies).

Mammalian expression vectors. The murine E3b1 cDNA used in this study was cloned from the Matchmaker mouse brain cDNA Library (Clontech Laboratories). It is identical to the sequence in the Database designated as Spectrin SH3 domain binding protein 1 (ssh3bp1; GenBank Accession No. BC004657). The coding region of murine E3b1 was subcloned into pcDNA3.1/MycHis vector (Invitrogen). pEBB/HA-PAK1 was provided by Dr. Bruce J. Mayer (University of Connecticut Health Center, Farmington, CT) and pcDNA3.1/MycHis-Rac1^{G12V} by Dr. J. H. Kim (Korea University, Seoul, Korea). Site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene), to obtain pEBB/HA-PAK1^{H83,86L}. The following mutagenic primers were used: 5′-CCTTCAGATTTTGAACtCACAATTCaTGTCGGTTTTGATGCT-3′ and 5′-AGCATCAAAACCGACAtGAATTGTGaGTTCAAAATCTGAAGG-3′. Mismatches are indicated by lowercase letters. The mutation was verified by automatic DNA sequencing. The PAK1 autoinhibitory domain (AID: corresponding to aa 83–149 of hPAK1) and AID L107F have been previously described [15,16].

Antibodies. The coding region of murine E3b1 was subcloned into pGEX-4T-1 vector (Pharmacia). A glutathione *S*-transferase (GST)-E3b1 fusion protein encoded by pGEX-4T-1/E3b1 was expressed in bacterial cells and purified by affinity chromatography. Polyclonal antibodies against the purified GST-E3b1 (anti-E3b1 antiserum) were prepared by immunizing rabbits with GST-E3b1 fusion proteins. Anti-HA polyclonal antibody was from Upstate Biotechnology and anti-HA monoclonal antibody from Cell Signaling Technology. Anti-myc 9E10 was used in the form of hybridoma culture medium.

Western blot analysis. Cells were washed with PBS and lysed with SDS–lysis buffer (100 mM Tris, pH 6.8, 2% SDS, and 10% glycerol). Lysates were boiled for 5 min. Protein concentration was determined with the BCA reagent (Pierce). Equal amounts of protein were resolved by SDS–PAGE and transferred to a nitrocellulose membrane. After blocking, the blots were incubated with anti-E3b1 antibody (1000:1), anti-HA polyclonal antibody (1000:1), or anti-myc hybridoma culture medium (100:1), washed with PBS-T (0.1% Triton X-100 in PBS), incubated with horseradish peroxidase-conjugated secondary antibody (Jackson Immuno Research Laboratories), and analyzed by ECL (Amersham).

Immunocytochemistry. Cells cultured in 35-mm dishes were transfected on 0.1% gelatin-coated coverslips for 24 h. For serum starvation, the cells were switched to serum-free medium for 10 min. They were then fixed for 10 min in 3.7% paraformaldehyde solution in phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100 in PBS for 5 min. Thereafter, they were blocked for 30 min in 0.1% Triton X-100 in PBS with 10% goat serum (Vector Laboratories) and 3% bovine serum albumin. The coverslips were then incubated with anti-E3b1 antibody (200:1) and/or anti-HA monoclonal antibody (200:1) for 1 h. After washing, they were stained with fluorescein-conjugated anti-mouse IgG and/or rhodamine-conjugated anti-rabbit IgG (100:1, Jackson ImmunoResearch Laboratories) for 1 h. Washed coverslips were mounted and observed with a Zeiss fluorescence microscope equipped with a 63× (1.4 NA) Planapochromat objective lens.

Results

E3b1 is rapidly degraded by calpain in serum-starved cells

E3b1 is widely expressed in tissues and cell lines [6,7]. We examined its stability in various cell lines in the absence of serum-factor stimulation. When Rat2 cells were incubated in DMEM lacking serum, the E3b1 protein level decreased markedly (Fig. 1A); about 80–90% was degraded in 10 min. The level recovered rapidly when cells were stimulated with 10% FBS (Fig. 1B). Both degradation and recovery of the protein were very rapid and detectable within 2 min (data not shown). The destruction of E3b1 proteins upon serum removal was not confined to Rat2 cells. We observed essentially the same phenomenon in NIH3T3 cells, a mouse fibroblast cell line; COS7 cells, a monkey kidney epithelial cell line; A431 cells, a human epidermoid carcinoma cell line; and MCF7 cells, a human breast cancer cell line (data not

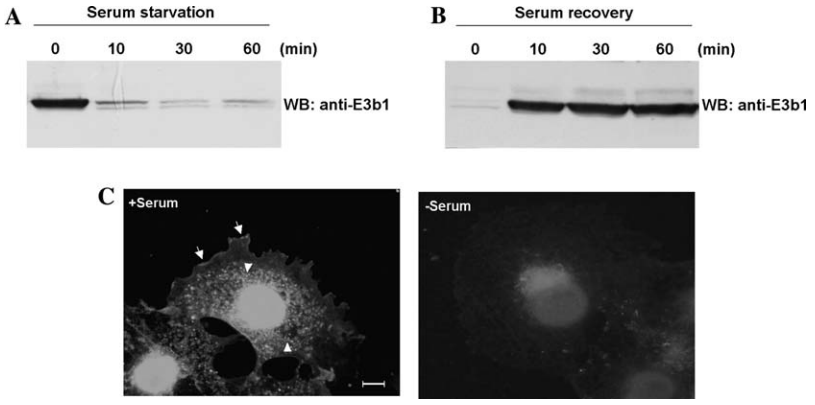

Fig. 1. Serum starvation induces rapid loss of E3b1 protein in Rat2 cells. (A) Cells grown in 10% FBS were serum-starved for the indicated times. (B) Cells grown in serum-free medium were re-supplied with 10% FBS for the indicated times. Equal amounts of lysates were analyzed by Western blotting (WB) with anti-E3b1 antibody. Data shown in (A) and (B) are representative of three independent experiments. (C) Cells grown in 10% FBS (+serum) were serum-starved for 10 min (−serum), fixed, and stained with anti-E3b1 antibody. Arrows indicate membrane ruffles and arrowheads vesicle-like structures. Scale bar, 10 μm. The cells in each panel are representative of three independent experiments.

shown). Destruction of E3b1 upon serum removal was confirmed by immunocytochemistry of Rat2 cells: in the presence of serum the cells exhibited prominent E3b1-staining at membrane ruffles and in small vesicle-like structures (Fig. 1C, +serum, arrows and arrowheads) and staining at these sites disappeared within 10 min of serum removal (Fig. 1C, −serum).

Because E3b1 contains multiple PEST motifs and these have been found in many proteins that are degraded by calpain or the ubiquitin-dependent 26S proteasome pathway, we tested whether the down-regulation of E3b1 was due to proteolysis by these protease systems. Serum-deprived Rat2 cells were exposed to inhibitors of calpain and the proteasome pathway for 12 h and E3b1 levels were examined. The degradation of E3b1 was greatly inhibited by the chemical calpain inhibitors ALLN (calpain inhibitor I, CI I), ALLM (calpain inhibitor II, CI II), E-64, and calpeptin, as shown in Fig. 2. Although ALLN and ALLM are calpain inhibitors they also act as proteasome inhibitors [17,18], and E-64 can inhibit other cysteine proteases. However,

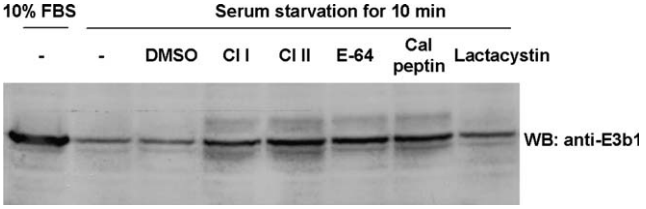

Fig. 2. Calpain inhibitors reverse E3b1 degradation induced by serum starvation. Rat2 cells were grown in 10% FBS in the presence or absence of the indicated inhibitors: DMSO (control), 25 μM calpain inhibitor I (CI I), 25 μM calpain inhibitor II (CI II), 100 μM E-64, 25 μg/ml calpeptin, or 25 μM lactacystin for 12 h and serum-starved for 10 min. Equal amounts of lysates were analyzed by Western blotting (WB) using anti-E3b1 antibody. Data shown are representative of three independent experiments.

the similar effect of the calpain-specific inhibitor, calpeptin, suggests that all these effects are mainly due to calpain inhibition. Moreover, the proteasome-specific inhibitor lactacystin did not significantly block the degradation of E3b1 (Fig. 2). We confirmed that E3b1 is a substrate for calpain, by showing that calpain could cleave in vitro-translated, [^{35}S]methionine-labeled E3b1 (data not shown).

Activated Pak1 inhibits degradation of E3b1

Protein phosphorylation has been suggested as a regulatory mechanism of calpain-dependent proteolysis [12]. Since over-expression of E3b1 activates Rac and its downstream effector kinase, Pak [3], we tested whether expression of Pak modulates the degradation of E3b1. COS7 cells were transiently transfected with wild type Pak1 or constitutively active Pak1^{H83,86L}. Over-expression of wild-type Pak1 did not affect the down-regulation of E3b1 upon serum starvation (Fig. 3A) and this was confirmed immunocytochemically (data not shown). In contrast, over-expression of Pak1^{H83,86L} did inhibit degradation (Fig. 3B). In view of the fact that only about 20% of the COS7 cells received Pak1^{H83,86L} in the transfection, this inhibition of E3b1 degradation was significant. Immunocytochemistry of the transfected cells confirmed that E3b1 was not degraded upon serum starvation in the cells transfected with Pak1^{H83,86L} (Fig. 3C, arrows and arrowheads). E3b1 and Pak1^{H83,86L} colocalized in membrane ruffles and in the vesicle-like structures. Interestingly, the small vesicle-like structures in the cells previously shown in Fig. 1C (+serum) where no Pak1^{H83,86L} was expressed seemed to fuse to form large vacuolar structures in the Pak1^{H83,86L}-expressing cells (Fig. 3C, arrowheads). Inhibition of E3b1 degradation by Pak1^{H83,86L} suggests that the removal of PAK-activating growth factors might be a trigger for

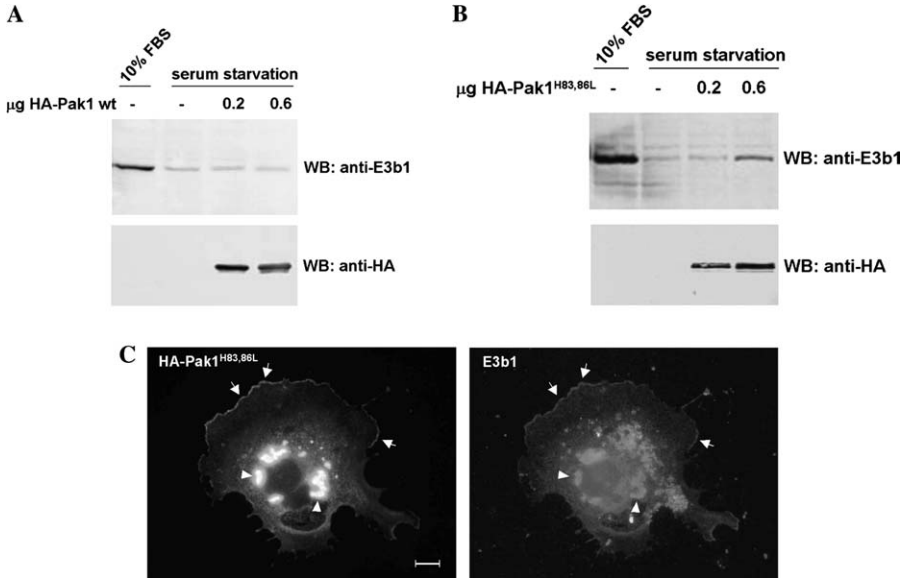

Fig. 3. Expression of the constitutively active form of Pak1, Pak1^{H83,86L}, inhibits degradation of E3b1 in serum-starved COS7 cells. (A,B) Cells were transiently transfected in 35 mm dish with the indicated amounts of HA-wild type Pak1 (A) or the constitutively active mutant, HA-Pak1^{H83,86L} (B). After 24 h, cells were serum-starved for 10 min. Equal amounts of lysate were analyzed by Western blotting (WB) with anti-E3b1 antibody. Data shown are representative of three independent experiments. (C) Cells were transiently transfected with HA-Pak1^{H83,86L}. After 24 h, cells were serum-starved for 10 min, fixed, and double-stained for HA-Pak1^{H83,86L} and E3b1 with anti-HA monoclonal antibody and anti-E3b1 antibody. Scale bar, 10 μm. In each panel the cells are representative of three independent experiments.

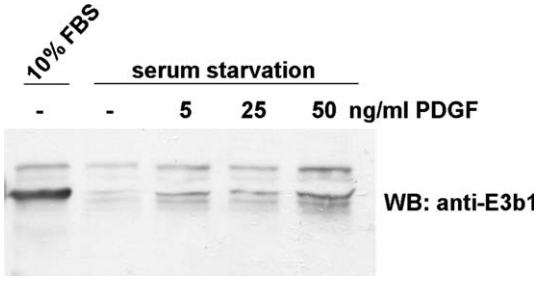

Fig. 4. Serum starvation-induced down-regulation of E3b1 in NIH3T3 cells is partially reversed by PDGF. Cells grown in 10% FBS were serum-starved for 10 min with or without PDGF-BB at the indicated concentrations. Equal amounts of lysate were analyzed by Western blotting (WB) with anti-E3b1 antibody. Data shown are representative of three independent experiments.

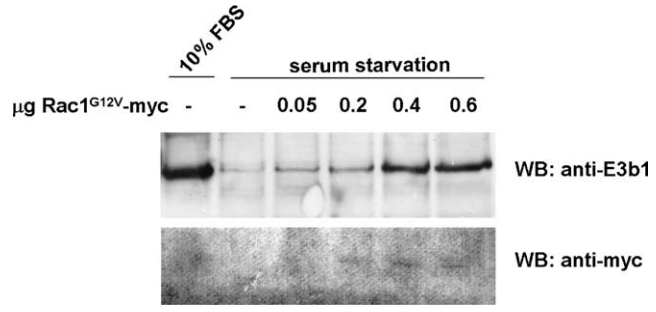

Fig. 5. Expression of the constitutively active form of Rac1, Rac1^{G12V}, inhibits down-regulation of E3b1 in COS7 cells. Cells were transiently transfected in 35 mm-dish with increasing amounts of the constitutively active Rac1 mutant, Rac1^{G12V}-myc. After 24 h, they were serum-starved for 10 min. Equal amounts of lysates were analyzed by Western blotting (WB) with anti-E3b1 antibody or anti-myc antibody. Data shown are representative of three independent experiments.

degradation. In order to examine whether activation of endogenous Pak by growth factors can protect E3b1 from degradation, we incubated NIH3T3 cells in DMEM supplemented with the known Pak-activating ligand, PDGF [19], and found that the loss of E3b1 protein was partially inhibited (Fig. 4). Higher concentrations of the growth factor were more protective, and the limited extent of protection may be due to the transient nature of the activation of downstream signaling by the native ligand. Rac1 is a well-known mediator of PAK activation in PDGF stimulation. We therefore also tested the effect of the constitutively active form of Rac1, Rac1^{G12V}. As expected, expression of Rac1^{G12V} inhibited the degradation of E3b1 (Fig. 5), whereas over-expression of the dominant-negative form of Rac1, Rac1^{T17N}, did not (data not shown). These observations indicate that Pak activation is required for the inhibition of E3b1 degradation and imply that the E3b1 level in the cell is maintained by feedback by the activated forms of its own downstream effectors, Rac and Pak.

Pak1 kinase activity regulates the stability of over-expressed E3b1

Interestingly, unlike the endogenous E3b1, the over-expressed E3b1 was not degraded upon serum

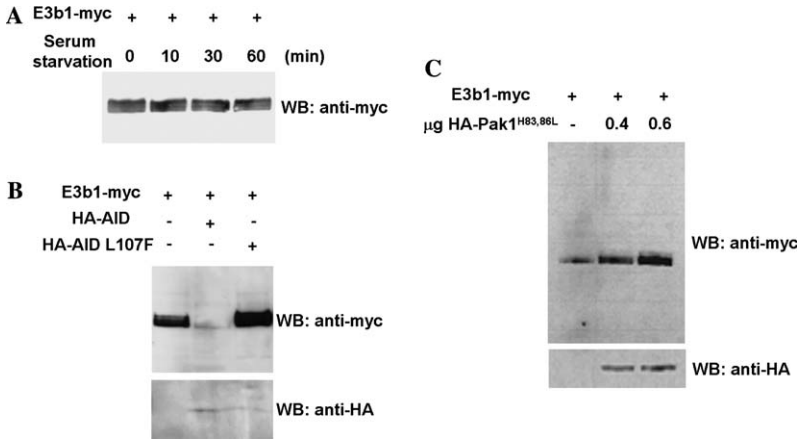

Fig. 6. Pak regulates stability of over-expressed E3b1 in COS7 cells. (A) Cells were transiently transfected with E3b1-myc. After 24 h, the cells were serum-starved for the indicated lengths of time. (B,C) Cells were transiently co-transfected as indicated with E3b1-myc and HA-AID or HA-AID L107F (B), or E3b1-myc and the indicated amounts of HA-Pak1^{H83,86L} in 35 mm dishes (C). The cells were maintained in 10 % FBS-containing media for 24 h. Equal amounts of lysate were analyzed by Western blotting (WB) with anti-myc or anti-HA antibody. For each panel, the data shown are representative of three or four independent experiments.

starvation (Fig. 6A). It has been reported by Innocenti et al. [3] that over-expression of E3b1 in COS7 cells results in increased PAK activity. We reasoned that the activation of PAK induced by over-expression of E3b1 might contribute to the stabilization of E3b1. To test this idea we examined the effect of expressing the PAK1 autoinhibitory domain (AID), which can block the kinase activity of PAK [15], on the stability of over-expressed E3b1. AID was co-expressed with E3b1 in the COS7 cells and they were maintained in the presence of serum. As shown in Fig. 6B, the over-expressed E3b1 proteins were degraded in the AID-expressing cells. They were unaffected when inactive AID (AID L107F) was co-expressed. In addition, over-expression of the active Pak1 mutant, Pak1^{H83,86L}, caused accumulation of over-expressed E3b1 (Fig. 6C), indicating that the increased activation of PAK1 can further protect E3b1 from degradation. These results demonstrate that the stability of over-expressed E3b1 can also be regulated by Pak kinase.

Discussion

In this study, we show that E3b1 is rapidly degraded upon serum starvation and stabilized by Pak1 activation. The presence of multiple PEST motifs in the E3b1 sequence prompted us to investigate the involvement of calpain and/or the ubiquitin-dependent proteasome pathway in the degradation of E3b1 and we found that E3b1 degradation was blocked by calpain inhibitors but not by the proteasome-specific inhibitor, lactacystin. In contrast, Dai et al. [9] reported that Abi-2, a highly related protein, is a substrate for ubiquitin-dependent degradation by the 26S proteasome. However, more recently, the same group reported that E3b1/Abi-1 is relatively resistant to Bcr-Abl-induced ubiquitin-dependent degradation [10]. The degradation of endogenous E3b1 by calpain rather than by ubiquitin-dependent proteasome machinery suggests that the cellular levels of E3b1/Abi-1 and Abi-2 proteins are affected by different regulatory mechanisms. E3b1/Abi-1 and Abi-2 are highly related genes and their products share 69% identity in their amino acid sequences. Although the function of Abi-2 is not clear, it may function as an antagonist of E3b1/Abi-1 by competing with it for tricomplex formation with Eps8 and Sos-1 [10]. If that is the case, the degradation of E3b1/Abi-1 or Abi-2 by different mechanisms could affect the strength of RTK-mediated Rac signaling depending on which mechanism is prevalent at a particular time.

Depletion of growth factors brought about by serum starvation could be a trigger for the destruction of E3b1 by the activation of calpain. This idea is supported by the observation that E3b1 degradation was partially reversed by PDGF (Fig. 4). There is a report on the participation of calpain rather than the proteasome in the degradation of cyclin D1 in serum-starved NIH3T3 cells [20]. Calpain activation is also known to be involved in myoblast differentiation in low levels of serum [21,22]. Calpains are Ca^{2+}-dependent cysteine proteases [23] whose activity is believed to be controlled by Ca^{2+} concentration [24]. Calpain-dependent degradation of E3b1 implies that the cellular signaling pathways controlling intracellular Ca^{2+} concentration [25–27] influence cellular levels of E3b1.

We have presented evidence that E3b1 is stabilized by its own downstream effector kinase (Figs. 3 and 6). This feedback regulation may allow for sustained Rac activation by stabilizing the level of E3b1 required for forming the Rac-activating Sos-1–E3b1–Eps8 tricomplex. How PAK activation leads to E3b1 stabilization is

not known. It is possible that PAK-dependent phosphorylation of calpain may regulate its activity. Both m- and μ-calpain are phosphorylated in vivo at serines and threonines as well as tyrosines and phosphorylation of Ser369/Thr370 of m-calpain by cAMP-dependent protein kinase A inhibits its activity [28]. It is also possible that PAK-dependent phosphorylation of E3b1 decreases its calpain-sensitivity. The E3b1-binding partner, Abl, is known to associate with PAK [29]. Therefore, E3b1 may interact with PAK on an Abl scaffold. Colocalization of E3b1 and the active form of Pak1, $Pak1^{H83,86L}$, in membrane ruffles and vesicle-like structures (Fig. 3C) also suggests that E3b1 is a substrate for active PAK1.

We have identified calpain-dependent degradation of E3b1 upon serum starvation in several cell lines, and demonstrated that activation of Rac-PAK downstream of E3b1 prevents degradation. Our findings suggest that E3b1 levels are controlled by two antagonistic mechanisms; one, involving calpain activation, causes down-regulation of E3b1, the other, involving PAK activation, stabilizes cellular levels of E3b1. Because E3b1 plays an essential role in RTK-mediated actin cytoskeletal reorganization by activating Rac, modulation of E3b1 levels may be an important mechanism affecting many cellular processes that require actin remodeling. Many influences affect the activation of calpain and PAK. In addition to intracellular Ca^{2+} flux, phospholipid binding, phosphorylation, and protein co-activators and inhibitors are possible calpain regulators [28,30]. PAK activity is also regulated by a variety of receptor-mediated signals and serum factors including PDGF [19,31]. The model in Fig. 7 suggests that the degradation of E3b1 by calpain could be the point of convergence of a variety of cellular signals that modulate the strength of RTK-mediated Rac signaling by regulating E3b1 levels.

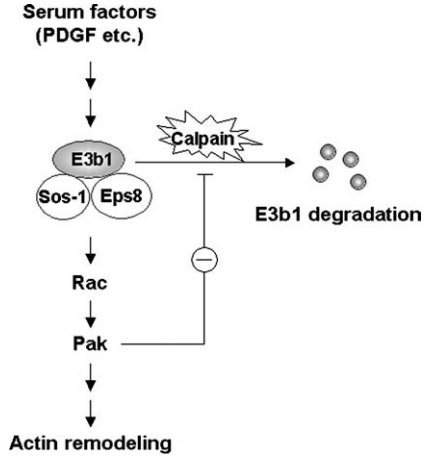

Fig. 7. A model of regulation of E3b1 levels by PAK. Serum factors (PDGF etc.) activate Rac/PAK signaling that regulates actin dynamics. At the same time, PAK activation inhibits calpain-dependent degradation of E3b1 and stabilizes E3b1 levels. This may allow activation of Rac signaling by stabilizing the Sos-1–E3b1–Eps8 tricomplex. In contrast, removal of serum factors induces rapid loss of E3b1 proteins by eliminating the inhibitory role of PAK activation on the calpain-dependent degradation of E3b1. The loss of E3b1 proteins terminates Rac/PAK signaling.

Acknowledgments

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