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The Cbl proteins are binding partners for the Cool/Pix family of p21-activated kinase-binding proteins

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Abstract Members of the Cool protein family contain SH3, Dbl, and pleckstrin homology domains and are binding partners for the p21-activated kinase (PAK). Using the yeast two-hybrid screen, we identified Cbl-b as a Cool family binding partner. We co-immunoprecipitated endogenous Cool and Cbl-b from a variety of breast cancer cell lines. The Cool–Cbl-b interaction requires the SH3 domain of Cool and competes with the binding of PAK to Cool proteins. Expression of Cbl-b effectively blocks the ability of Cool-2 to stimulate PAK, thus providing an additional mechanism, aside from catalyzing receptor ubiquitination, by which Cbl-b acts as a negative regulator for signaling activities requiring PAK activation.

Key words: SH3 domain; Cbl; Breast cancer cell; Cdc42; p21-activated kinase

1. Introduction

A number of lines of evidence indicate that growth factor-coupled signaling pathways utilize GTP-binding proteins which serve as molecular switches to activate specific target/effector activities. In addition to the well known coupling between receptor tyrosine kinases and Ras, it has recently become appreciated that members of the Rho subfamily, and in particular Cdc42 and Rac, are sensitive to growth factor activation and are able to initiate signaling cascades that lead to the nucleus [1–3]. One of the best known targets for Cdc42 and Rac are members of the p21-activated kinase (PAK) family of serine/threonine kinases [4–7]. Stimulation of PAK by Cdc42/Rac can trigger a series of signaling events that culminate in the activation of stress-responsive mitogen-activated protein (MAP) kinases, including the c-Jun kinase and p38 [8–12]. PAK activity has also been implicated in various actin cytoskeletal rearrangements and in alterations in cell morphology, thus providing a potential control point for linking signals to the nucleus with those influencing cell shape and motility [13–16].

Given the fact that PAK is a predominant signaling target/effector for Cdc42 and Rac, and because it apparently can influence a number of cellular activities, it seemed likely that PAK activity would be tightly regulated in cells. In order to learn more about the underlying mechanisms responsible for the regulation of PAK activity, as well as gain additional insights into the signaling pathways utilizing PAK, we set out to search for PAK-binding partners. This led us to a family of proteins that we originally named Cool (for cloned out of library [17]). The Cool proteins all contain an SH3 domain followed by Dbl homology and pleckstrin homology (PH) domains. We have characterized three members of the family in detail, the first being a 50 kDa protein, p50Cool-1, which binds via its SH3 domain to a stretch of proline residues within the amino-terminal regulatory half of the PAK molecule and acts as a negative regulator of PAK activity [17]. A somewhat longer splice variant form of p50Cool-1, designated p85Cool-1 (identical to β-Pix for PAK-interactive exchange factor [18]) and p85SPR [19], neither inhibits nor directly activates PAK, while a third related protein, p90Cool-2 (identical to α-Pix [18] and referred to from here on as Cool-2), strongly stimulates PAK activity as a result of its catalyzing the guanine nucleotide exchange activity of Rac (Feng et al., unpublished data), as well as possibly through its direct binding to the kinase [20].

In this work, we show that members of the Cbl (Casitas B lymphoma) family of proteins represent a new class of Cool-binding partners. The c-Cbl protein represents the cellular homolog of the viral transforming protein of the murine Cas NS-1 retrovirus, which contains the amino-terminal 355 amino acids of c-Cbl, including a nuclear localization signal [21]. It is also ~55% identical to the *Caenorhabditis elegans* sli-1 gene product, which antagonizes the actions of the let-23 gene product (i.e. the epidermal growth factor (EGF) receptor homolog) in the signaling pathway responsible for vulval development [22]. It was originally suspected that c-Cbl might have a hematopoietic-specific function, as it was shown to be tyrosine phosphorylated in Jurkat T cells upon T cell receptor activation, as well as transiently tyrosine phosphorylated in macrophages after colony-stimulating factor-1 stimulation [23,24]. However, it was subsequently shown to be tyrosine phosphorylated in an EGF-dependent manner and thought to serve as some type of scaffold in EGF-coupled signaling, given

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Abbreviations: PAK, p21-activated kinase; Cool, cloned out of library; Pix, PAK-interactive exchange factor; Cbl, Casitas B-lymphoma

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that it was composed of a number of distinct domains including a nuclear localization sequence, as well as SH2, ring-finger, leucine-zipper and proline-rich domains.

Recently, it has also been reported that the Cbl proteins have E3 ubiquitin ligase activity and participate in the degradation of EGF receptors [25,26], which may at least in part contribute to their negative regulatory effects on EGF receptor-coupled signaling. Here, we demonstrate that the c-Cbl homolog, Cbl-b, which was originally cloned from a breast cancer cell library and is enriched in breast cancer cells [27], forms a stable complex with the Cool proteins through their common SH3 domain. We show that Cbl-b competes with PAK for binding to the Cool proteins and that this results in an inhibition of PAK activity. Thus, the ability of Cbl-b to bind the Cool/Pix proteins offers an additional mechanism by which it can negatively regulate EGF receptor-coupled signaling activities, by inhibiting the Cool-2/α-Pix-mediated activation of PAK and its ability to signal downstream of the stress-responsive nuclear MAP kinases.

2. Materials and methods

2.1. Cell culture, antibodies and other reagents

MDA-MB 453 and 468 human mammary carcinoma cells were kindly provided by Dr. Mark Sliwkowski (Genentech). SKBR3, BT20, and MDA-MB-231 human mammary carcinoma cells were purchased from ATCC. All cell lines were grown in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. Myc-tagged p85Cool-1 and the SH3-binding-defective mutant Myc-p85Cool-1(W43K) were prepared as described in Bagrodia et al. [17]. The hemagglutinin (HA)-tagged Cbl-b construct was a kind gift from Dr. Stan Lipkowitz (National Cancer Institute, Bethesda, MD, USA). The HA-tagged c-Cbl construct was prepared by inserting the cDNA encoding c-Cbl (a kind gift from Dr. Wallace Langdon, Queen Elizabeth Medical Center, Australia) into pGEM4Z. The Cbl-b construct lacking the proline-rich domain (residues 1–418; designated Cbl-b-RF) was prepared using the polymerase chain reaction (PCR), followed by subcloning the PCR product into HA-pcDNA3. The PAK3 construct lacking the kinase domain (residues 1–327; designated PAK(KD)) was also generated by PCR and subcloning into HA-pcDNA3.

Anti-c-Cbl and anti-Cbl-b antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Cool antibody was prepared using hexa-histidine (His)-tagged p50Cool-1 that was treated with thrombin to remove the His tag and then purified on a Mono Q-Sepharose column [17]. HA-tagged proteins were immunoprecipitated and/or Western blotted with monoclonal antibody (mAb) 12CA5 ascites or CA-11 (Babco), and Myc-tagged proteins were immunoprecipitated and/or Western blotted with mAb 9E10.

2.2. Yeast two-hybrid screen

The yeast strain L40 was co-transformed with p50Cool-1 fused to the LexA DNA-binding domain, which regulates expression of both his3 and lacZ, and a Jurkat cDNA library fused with the Gal4 activation domain [28]. Clones positive for β-galactosidase activity were rescued, and a partial clone was sequenced and shown to be identical to a region within the carboxy-terminal half of the Cbl-b protein (see Section 3).

2.3. Cell lysis and kinase assays

Cells were washed with cold phosphate-buffered saline, and lysed in 20 mM HEPES, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM sodium vanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The lysates were then centrifuged at 12 000 rpm for 25 min at 4°C.

PAK3 assays were performed as follows. The amounts of PAK3 in the lysates were determined by Western blotting. Lysates containing essentially equivalent amounts of PAK3 were immunoprecipitated with an anti-PAK3 primary antibody (a gift from Dr. S. Pelech, Kinetek Biotechnology, Vancouver, BC, Canada) for 1–2 h followed by incubation with protein A-Sepharose for an additional hour. Immunoprecipitates were washed (three times) with lysis buffer and then with 2×kinase buffer (40 mM HEPES, pH 7.4, 20 mM $MgCl_2$, and 4 mM $MnCl_2$). Myelin basic protein (MBP; Sigma) was then used as a phosphorylation substrate (5 μg). The kinase reactions were initiated by the addition of kinase buffer and 20 μM [γ-^{32}P]ATP (3000 Ci/mmol) for 5 min at room temperature. The kinase reactions were stopped by the addition of 2×sodium dodecyl sulfate (SDS) sample buffer containing 20 mM EDTA. Proteins eluted by boiling for 2–5 min were resolved by 12.5% SDS–polyacrylamide gel electrophoresis. The proteins were transferred to Immobilon P membranes (Millipore) and autoradiographed prior to immunoblotting. The blots were probed with anti-HA, anti-Myc, anti-Cool, or anti-PAK3 antibodies. The primary antibodies were detected with horseradish peroxidase-coupled sheep anti-mouse or donkey anti-rabbit antibody by ECL (Amersham Pharmacia Biotech).

3. Results and discussion

We have used the yeast two-hybrid screen to identify binding partners for the PAK and Cool proteins. The Cool proteins (p50Cool-1 and p85Cool-1) were originally identified from this screen as binding partners for PAK3 [17]. We then isolated a partial cDNA from a Jurkat cell cDNA library that appeared to encode a binding partner for p50Cool-1. Data base analyses indicated that this cDNA fragment encoded a peptide that matched a portion of the proline-rich domain of Cbl-b (Fig. 1), a member of the Cbl family that was originally cloned from a ZR75-1 breast cancer cell cDNA library [27]. Cbl-b is about 60% identical to c-Cbl and shares all of the same functional motifs.

Based on this initial indicator from the yeast two-hybrid screen, we then set out to determine whether Cbl-b binds to the Cool proteins in mammalian cells. We used a specific antibody raised against Cbl-b to immunoprecipitate the endoge-

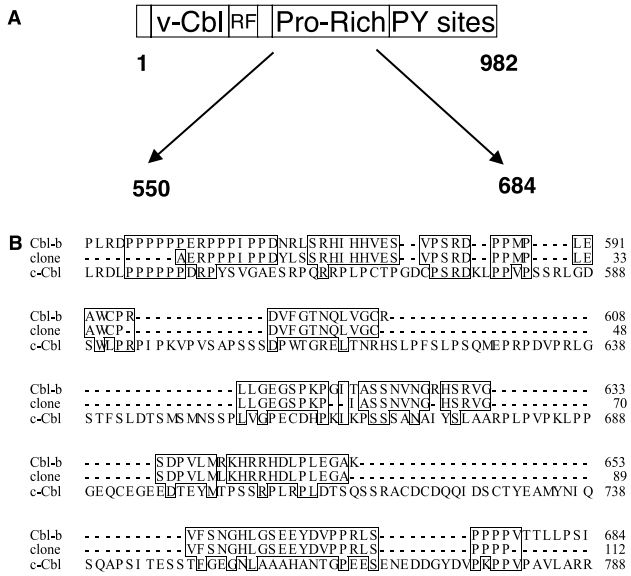

Fig. 1. Yeast two-hybrid screen identified Cbl-b as a potential binding partner for the Cool proteins. A: Depiction of the different domain structures of Cbl-b. Both Cbl-b and c-Cbl contain the limit region that comprises the viral Cbl protein (v-Cbl), as well as ring-finger (RF) and proline-rich (Pro-rich) domains, and carboxy-terminal tyrosine phosphorylation (PY) sites. B: Comparison of the sequence deduced from the partial clone corresponding to the Cool-binding protein with the corresponding sequences for human Cbl-b and c-Cbl. Regions of amino acid identity are included within boxes. Alignment was achieved using the Clustal X program.

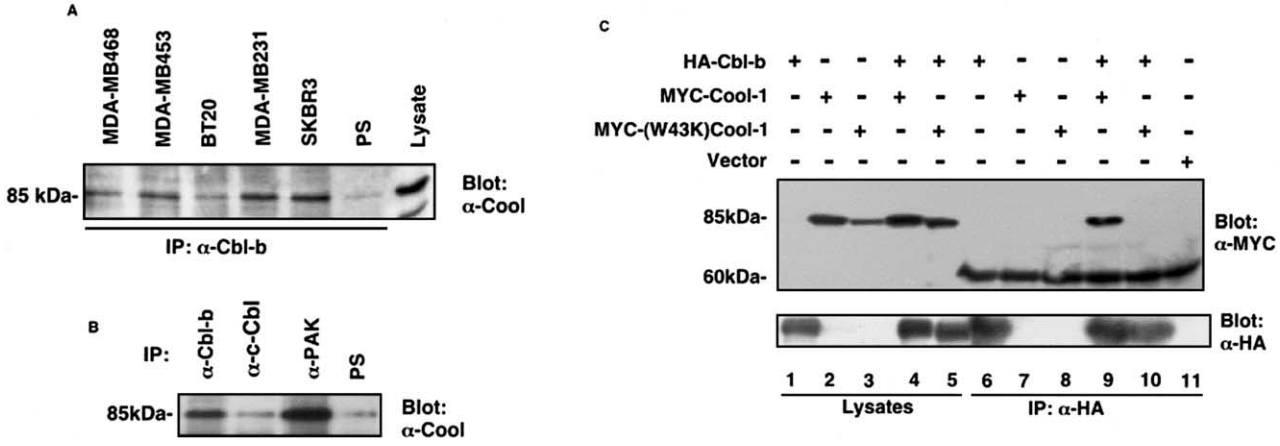

Fig. 2. Cbl-b binds Cool in human breast cancer cells. A: Endogenous Cbl-b was immunoprecipitated from the lysates of the different indicated human breast cancer cells using a specific anti-Cbl-b monoclonal antibody. The final lane represents 10% of the whole cell lysates from SKBR3 cells that was used for immunoprecipitation. PS represents pre-immune serum (control). The proteins were Western blotted and the blots were probed with an antibody raised against p50Cool-1. Based on molecular weight standards, the band recognized by the anti-Cool antibody has an apparent M_r of 85 kDa and thus corresponds to endogenous p85Cool-1/β-Pix. B: Endogenous Cbl-b, c-Cbl, and PAK were immunoprecipitated from SKBR3 cells and assessed for the binding of endogenous p85Cool-1 by Western blotting using the anti-Cool antibody. PS represents the pre-immune serum control. C: The SH3 domain of the Cool proteins is essential for binding Cbl-b. COS cells were transfected with plasmids expressing the indicated HA- or Myc-tagged proteins. Anti-HA immunoprecipitates (12CA5) (lanes 6–11, main panel) were Western blotted with anti-Myc (9E10) to detect the presence of p85Cool-1. Lanes 1–5 represent 10% of the whole cell lysates used in the immunoprecipitation reactions. The lower panel is an anti-HA blot (for HA-Cbl-b). The bands at ~60 kDa in the immunoprecipitation experiments represent IgG.

nous protein from a variety of human breast cancer cell lines and then assayed for the presence of endogenous Cool by Western blotting with rabbit antiserum that was raised against the Cool-1 proteins [17]. As shown in Fig. 2A, we detected a specific interaction between Cbl-b and p85Cool-1 in every cell type examined. We did not find p50Cool-1 in the Cbl-b immunoprecipitates, probably because it is typically present in much lower abundance and is difficult to detect relative to p85Cool-1 [17].

Given the sequence similarity between Cbl-b and c-Cbl, it was of interest to examine whether c-Cbl was also capable of associating with the Cool proteins. When similar types of immunoprecipitation experiments were performed in human breast cancer cells using an anti-c-Cbl antibody for immunoprecipitation, followed by Western blotting with the anti-Cool antiserum, we typically did not detect a specific interaction between c-Cbl and p85Cool-1 (Fig. 2B). In some experiments, we did observe the co-immunoprecipitation of c-Cbl with p85Cool-1, although this was infrequent. Thus, c-Cbl is apparently a weaker binding partner for the Cool proteins compared to Cbl-b, such that in human breast cancer cells Cbl-b outcompetes c-Cbl for binding Cool. However, when Myc-tagged p85Cool-1 and HA-tagged c-Cbl were co-expressed in NIH 3T3 cells (which lacked Cbl-b), we were able to co-immunoprecipitate p85Cool-1 and c-Cbl (Wu et al., submitted).

Because the initial protein sequence obtained from our yeast two-hybrid screen overlapped the proline-rich domain of Cbl-b (Fig. 1), we examined whether the interactions between Cbl-b and the Cool proteins were mediated through the Cool SH3 domain. The results presented in Fig. 2C suggest that this is in fact the case. When a conserved tryptophan residue within the SH3 domain of p85Cool-1 was mutated to a lysine residue, the binding of Cbl-b to p85Cool-1 was completely abolished. Like Cbl-b, c-Cbl contains a stretch of proline residues and we have found that the p85Cool-1(W43K) mutant is also incapable of co-immunoprecipitating with HA-tagged c-Cbl in NIH 3T3 cells (Wu et al., submitted).

The requirement of a functional SH3 domain for binding the Cbl proteins indicates that each of the Cool proteins is capable of such an interaction, as they either share a common (p50Cool-1 and p85Cool-1) or highly similar (Cool-2) SH3 domain which they use to bind PAK [17]. This in turn suggests that the Cbl proteins should compete with PAK for binding the Cool proteins.

We examined potential competition between Cbl-b and PAK for the Cool proteins by determining the effects of Cbl-b on the Cool-2-mediated activation of PAK. While it was originally suggested that the Cool/Pix proteins acted as upstream activators of Cdc42/Rac [18], we had not been able to directly demonstrate nucleotide exchange activity for p50Cool-1 or p85Cool-1 [17,29]. We now know that this is due to the presence of a specific sequence of 18 amino acid residues that is immediately downstream of the PH domains of the Cool-1 proteins but is missing in Cool-2 [29]. Cool-2 is capable of acting as a guanine nucleotide exchange factor (GEF [29]) and we have recently found that it is a highly effective GEF for Rac in cells (Feng et al., unpublished data). This may explain how Cool-2 strongly stimulates PAK activity [29]. The Cool-2-mediated activation of PAK can be observed when immunoprecipitating PAK from COS cells and then assaying its ability to phosphorylate MBP. An example is shown in Fig. 3A, where Myc-tagged Cool-2 was co-immunoprecipitated with either HA-tagged full-length PAK3 or an HA-tagged PAK3 construct that lacks the kinase domain (designated PAK3(ΔKD)). Phosphorylation of MBP was only observed when full-length PAK3 was co-immunoprecipitated with Cool-2, and not when PAK3(ΔKD) was co-immunoprecipitated with the Cool-2 protein. Little or no phosphorylation of MBP occurred when PAK3 was expressed and assayed in the absence of Cool-2 (see Figs. 3C and 4B).

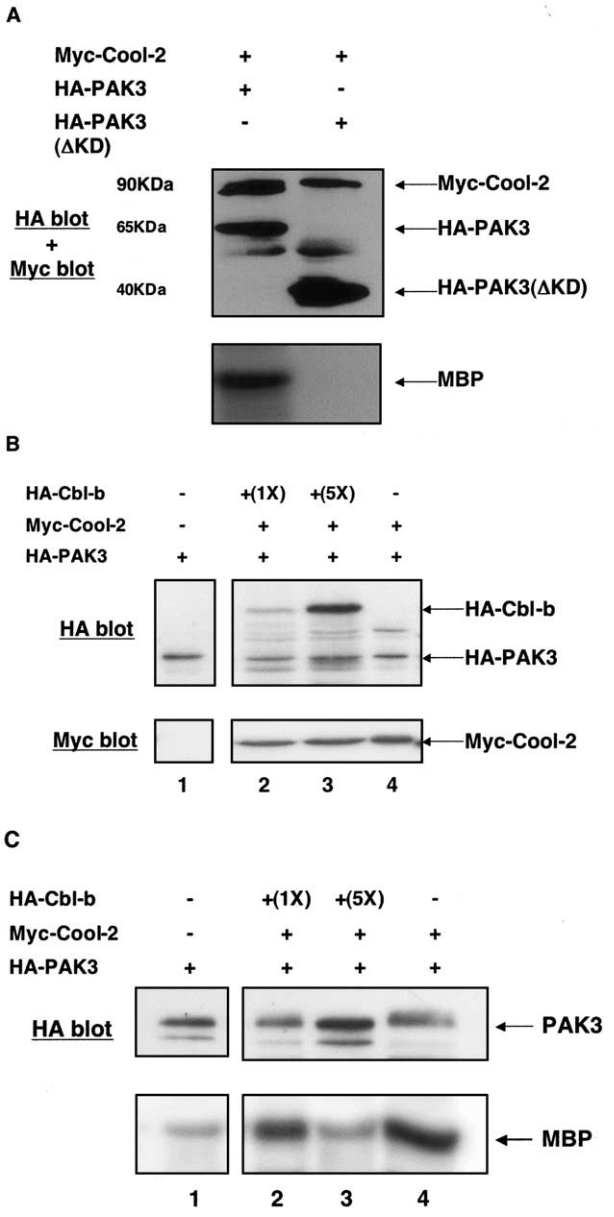

Fig. 3. Cbl-b inhibits Cool-2-stimulated PAK3 activity. A: COS cells were transiently transfected with HA-PAK3 (0.5 μg), HA-PAK3 that lacks the kinase domain (designated ΔKD; residues 1–327) (2 μg), and Myc-tagged Cool-2 (2 μg) as indicated. The upper panel represents the immunoprecipitation of the PAK constructs with anti-HA, followed by probing the blots with anti-HA, to detect the relative amounts of PAK, and with anti-Myc to detect Cool-2. The lower panel represents PAK activity measured in an immune complex kinase assay using MBP as a substrate. B: COS cells were transiently transfected with HA-PAK3 (0.5 μg), HA-Cbl-b (1.0 μg, lane 2; 5.0 μg, lane 3), and Myc-tagged Cool-2 (2 μg) as indicated. The upper panel was probed with anti-HA to detect the relative amounts of Cbl-b and PAK that were expressed, and the bottom panel of the blot was probed with anti-Myc to detect Cool-2. C: The upper panel shows the relative amounts of PAK3 assayed for the different conditions and the lower panel compares PAK activity assayed using MBP as substrate.

An interesting question then concerns whether Cool-2 must be able to bind PAK in order to mediate its activation. If so, the competitive binding of Cbl-b to PAK should prevent Cool-2 from stimulating PAK activity.

In Fig. 3C (lower panel), we show the results of PAK3 assays performed under conditions where the relative levels of Cbl-b to Cool-2 were significantly different. The expression of Cool-2 together with PAK3, but in the absence of Cbl-b, gave the expected strong stimulation of PAK activity (Fig. 3C, lower panel, lane 4), compared to cells expressing PAK3 alone (lane 1). In cells that expressed the lower level of Cbl-b relative to PAK3 (Fig. 3C, lane 2), there was no detectable effect on PAK activity compared to cells that were not transfected with Cbl-b, whereas in cells that expressed the higher level of Cbl-b relative to PAK3 (Fig. 3C, lane 3), the ability of Cool-2 to stimulate PAK activity was completely lost. Note that the inhibition by Cbl-b is clearly detected, despite the fact that a greater amount of PAK3 was assayed in this case (see the upper panel in Fig. 3C).

The results presented in Fig. 3 indicate that Cbl-b competitively inhibits the binding of Cool-2 to PAK. This also demonstrates that the binding of Cool-2 to PAK is necessary for Cool-2 to promote PAK activation. The ability of Cool-2 to stimulate the GEF activity of Rac, as assayed by the Cool-2-stimulated dissociation of [^{3}H]GDP from Rac, does not require the binding of PAK to Cool-2 (Feng et al., unpublished

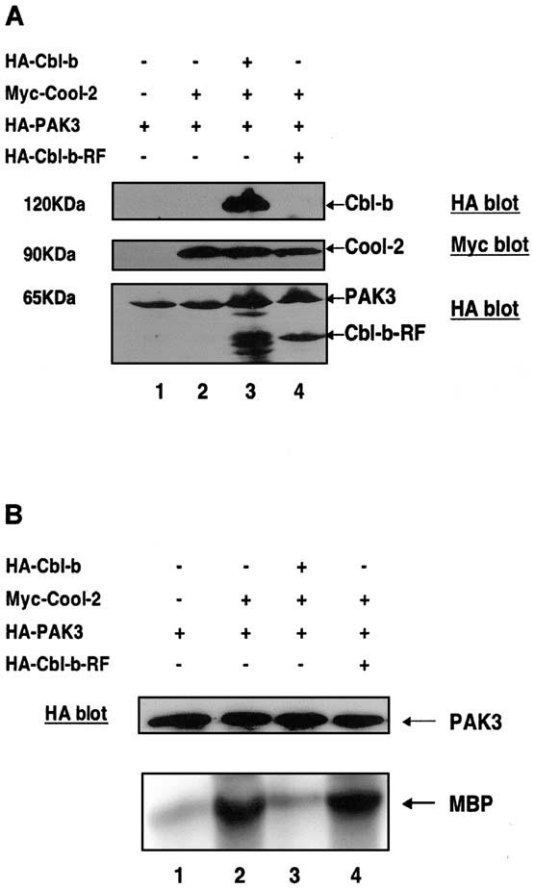

Fig. 4. A Cbl-b construct defective in binding Cool-2 does not inhibit Cool-2-stimulated PAK activity. A: COS cells were transiently transfected with HA-PAK3 (0.5 μg), Myc-tagged Cool-2 (2 μg), and either full-length Cbl-b (5 μg) or a Cbl-b construct (5 μg) that is truncated at the end of the ring-finger domain and lacks the proline-rich region responsible for binding Cool-2 (designated Cbl-b-RF, residues 1–418). The lysates were then blotted with anti-HA to detect PAK3, Cbl-b, and Cbl-b-RF, and with anti-Myc to detect Cool-2. The lower bands seen in the Cbl-b lane (i.e. lane 3) represent some degradation products from Cbl-b. B: PAK3 assays were performed as described in Fig. 3C.

data). Rather, the requirement for Cool-2 to bind to PAK in order to promote its activation might imply that PAK serves to recruit Cool-2 to the proper cellular location so that Cool-2-stimulated GDP–GTP exchange on Rac is directly translated into the stimulation of PAK activity by GTP-bound Rac.

When experiments were performed using a Cbl-b protein that lacks the Cool-binding site (i.e. the proline-rich domain), as originally identified in our yeast two-hybrid screens, Cool-2-mediated PAK activation was fully restored. Fig. 4 shows the results of such an experiment. Comparisons of the ability of Cool-2 to stimulate the PAK3-catalyzed phosphorylation of MBP in the presence of either full-length Cbl-b, or a Cbl-b construct that is truncated after the ring-finger domain and thus lacks the proline-rich domain, are shown in the lower panel of Fig. 4B (the upper panel compares the amount of PAK3 assayed for each condition). It is clear that while excess Cbl-b can strongly inhibit Cool-2-stimulated PAK activity (compare lanes 2 and 3 in the lower panel of Fig. 4B), the Cbl-b construct lacking the Cool-binding site shows no inhibitory effects (compare lanes 2 and 4).

Recently, it has been reported that the Cbl proteins have E3 ubiquitin ligase activity and participate in the degradation of EGF receptors [25,26], which may at least in part contribute to their negative regulatory effects on EGF receptor-coupled signaling. Here we provide a possible mechanism for an inhibitory signaling activity conferred by the Cbl proteins that is distinct from their ubiquitin ligase activity [30]. This occurs as an outcome of the direct binding of the Cbl proteins to Cool-2. Under those circumstances where the Rac GEF, Cool-2, is able to bind to PAK, a serine/threonine kinase and a primary signaling partner for Rac, a complex is formed that connects the activation of Rac directly to the stimulation of PAK activity. However, by competing with PAK for binding to Cool-2, the Cbl proteins disrupt this connection and block Rac activation and signaling through PAK. Thus, we suggest that the Cbl proteins have the potential to serve in a dual capacity to antagonize EGF receptor- or related tyrosine kinase-coupled signaling activities, through their abilities to catalyze receptor ubiquitination and to interfere with Cool-2/Rac-mediated PAK activation.

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