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Biochemical and Biophysical Research Communications 367 (2008) 183–189
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# Up-regulation of the Cbl family of ubiquitin ligases is involved in ATRA and bufalin-induced cell adhesion but not cell differentiation

Xiujuan Qu [1], Yunpeng Liu [1,*], Yanju Ma, Ye Zhang, Yingchun Li, Kezou Hou

*Department of Medical Oncology, The First Hospital, China Medical University, NO. 155, North Nanjing Street, Heping District, Shenyang City, Liaoning 110001, China*

Received 12 December 2007
Available online 27 December 2007

**Abstract**

The Casitas B-lineage Lymphoma (Cbl) family of ubiquitin ligases is multifunctional proteins that play important roles in different cell signaling pathways. It has been reported that c-Cbl and Cbl-b mRNAs are up-regulated during TPA-induced U937 and HL-60 cell differentiation. But the mechanism of the up-regulation and the roles of the Cbl family of ubiquitin ligases still remain unclear. In the present study, we demonstrated that bufalin enhanced all-*trans* retinoic acid (ATRA) induced differentiation of HL-60 cells, accompanied by up-regulation of the Cbl family of ubiquitin ligases. CsA, an inhibitor of calcium mobilization, reversed this up-regulation. Pretreatment with CsA and PS-341 did not affect the expression of CD11b, but suppressed the percentage of adherent cells. Lipid raft localization of Cbl-b enhanced cell adhesion, while C-terminal deletion partially suppressed the effect. Moreover, the expression of the adhesion-related kinases Pyk2 and Paxillin was up-regulated in parallel with the increase of Cbl proteins. These results suggested that up-regulation of c-Cbl and Cbl-b was involved in the regulation of ATRA and bufalin-induced HL-60 cell adhesion rather than cell differentiation, which might be mediated by lipid raft localization, ubiquitin ligase activity and C-terminal structure of Cbl proteins. Meanwhile, up-regulation of proline-rich tyrosine kinase (Pyk2) and Paxillin might also be implicated in this regulation.


*Keywords:* Cbl-b; c-Cbl; Ubiquitin ligases; Differentiation; Adhesion

Human myelocytic leukemia cells differentiate into myeloid cells when treated with ATRA [1]. However, ATRA monotherapy may be complicated by the emergence of a differentiation-resistant malignant cell population and retinoic acid syndrome [2,3]. Therefore, cooperative drugs have to be developed to circumvent these problems. Bufalin, one of the bufadienolides in the traditional Chinese medicine *Chan'su* [4], has multiple functions in cell apoptosis and differentiation [5–7]. We and others have shown that bufalin enhances retinoid-induced differentiation of acute myeloid leukemia (APL) cells from the patients and the NB4 cell line [8]. But the cooperative mechanisms still remain unclear.

The Cbl family proteins, including c-Cbl and Cbl-b, are members of a superfamily of RING finger E3 ubiquitin protein ligases. The Cbl family of ubiquitin ligases contains tyrosine kinase binding (TKB) and RING finger domains, a proline-rich region, several tyrosine phosphorylation sites and an ubiquitin-associated domain. The presence of a Cbl family member causes some important signaling molecules, such as Lyn, Syk, and Gab2, to be ubiquitinylated [9–13], and plays an important role in immune regulation, allergy and other cell functions. It has been reported that Cbl proteins can also function in cell differentiation. C-Cbl is activated in retinoic acid (RA) treated acute myeloid leukemia cell lines [14]. Phorbol 12-myristate 13-acetate (PMA) treatment of pre-osteoclastic leukemia FLG 29.1 cells induced a strong increase in the expression of c-Cbl and Pyk2, which affected the cellular adherent ability [15]. Both c-Cbl and Cbl-b mRNA are up-regulated during differenti-

* Corresponding author. Fax: +86 24 83282240.
*E-mail address:* liuyunpeng@medmail.com.cn (Y. Liu).
[1] These authors contributed equally to this paper.

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doi:10.1016/j.bbrc.2007.12.120

ation of U937 and HL-60 cells induced by TPA [16]. Therefore, up-regulation of the Cbl family of ubiquitin ligases may exhibit some role during the differentiation period, but the mechanism has not been sufficiently elucidated.

In the present study, we aimed to evaluate the roles of the Cbl family of ubiquitin ligases in the differentiation of a human promyelocytic cell line. We found that the expression of c-Cbl and Cbl-b was up-regulated in a time-dependent manner during ATRA and bufalin-induced HL-60 differentiation. However, the up-regulation of Cbl proteins did not affect cell differentiation, but might be involved in cell adhesion. Our study provides new insight to the Cbl family of ubiquitin ligases and their regulation of adhesion in differentiated HL-60 cells.

## Materials and methods

*Materials and antibodies.* Anti-Cbl-b and anti-phospholipase C-gamma2 (PLC-γ2) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-c-Cbl monoclonal antibody (mAb) was from Transduction Laboratories (Lexington, KY). Anti-tubulin monoclonal antibody was purchased from BD Biosciences Pharmingen (San Jose, CA). Anti-phosphotyrosine (4G10) agarose conjugate was obtained from Upstate Biotechnology Inc. (Lake Placid, NY).

*Cells and cell culture.* The human promyelocytic cell line HL-60 was grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS), penicillin (5 U/mL) and streptomycin (50 mg/mL) under a 95% air/5% $CO_2$ atmosphere. Rat basophilic leukemia RBL-2H3 cells and cells expressing different forms of Cbl-b in the lipid raft were maintained as monolayer cultures in Dulbecco's modified Eagle medium (DMEM) containing 100 U/mL penicillin and 10% heat-inactivated FBS. The transfected cells were cultured in the presence of 0.4 mg/mL G418 (Invitrogen, Carlsbad, CA).

*Determination of cell differentiation.* HL-60 cell differentiation was assessed by CD11b expression. Briefly, $2 \times 10^5$ cells treated with different reagents for indicated times were harvested by centrifugation and incubated with FITC-conjugated anti-CD11b antibody at 37 °C for 2 h in the dark, followed by washing with pre-chilled phosphate buffered saline (PBS) four times. Then the expression was analyzed by flow cytometry (FACS Calibur, Becton–Dickinson, Franklin Lakes, NJ). The mean expression of CD11b expressed in percentages ±SD of 3–6 experiments was shown.

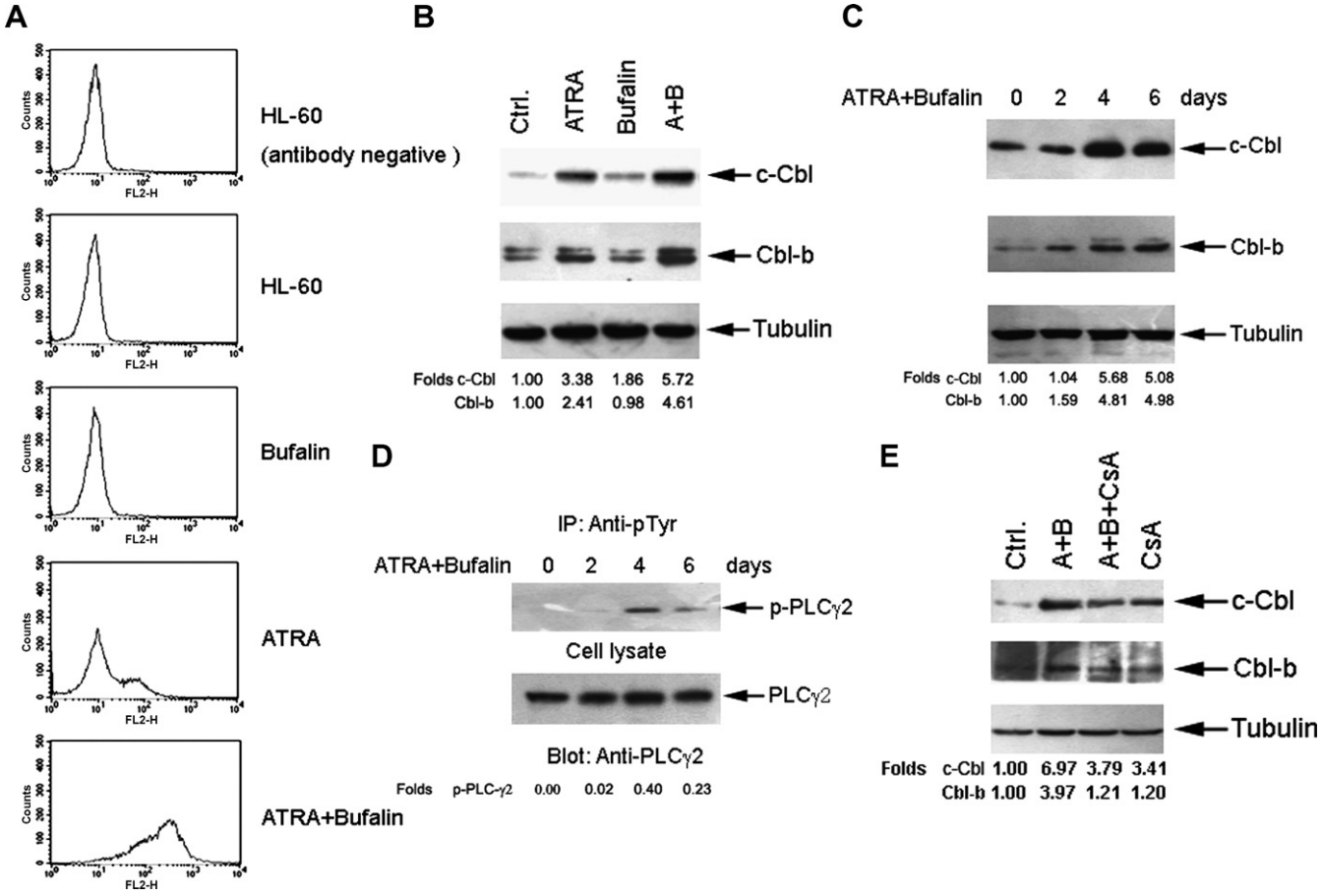


Fig. 1. Effect of bufalin on HL-60 cell differentiation induced by ATRA, and on the expression of the Cbl family of ubiquitin ligases. HL-60 cells were treated with 30 nM ATRA, 5 nM bufalin, or a combination of 30 nM ATRA and 5 nM bufalin for 4 d, respectively. The expression of CD11b was analyzed by FACS to determine cell differentiation (A). The expression of c-Cbl and Cbl-b was detected by Western blotting with anti-c-Cbl, anti-Cbl-b and anti-tubulin mAbs (B). HL-60 cells were treated with a combination of 30 nM ATRA and 5 nM bufalin for the indicated days. The expression of c-Cbl and Cbl-b was analyzed by Western blotting. (C) Cell lysates were precipitated with anti-phosphotyrosine agarose, and analyzed by Western blotting with anti-PLC-γ2 (D). HL-60 cells were pre-treated with 1 μM CsA for 2 h before exposure to the combination of 30 nM ATRA and 5 nM bufalin for 4 d. The expression of c-Cbl and Cbl-b was analyzed by Western blotting (E). *A* indicates ATRA; *B* indicates bufalin; *A* + *B* indicates the combination of ATRA and bufalin. The final results were analyzed with Image J software (NIH).

*Immunoprecipitation and immunoblotting.* Cells were washed twice with ice-cold PBS and solubilized in 1% Triton lysis buffer (1% Triton X-100, 50 mM Tris (tris (hydroxymethyl) aminomethane), pH 7.4, 150 mM NaCl, 10 mM EDTA (ethylenediaminetetraacetic acid), 100 mM NaF, 1 mM $Na_3VO_4$, 1 mM phenylmethylsulfonyl fluoride, and 90 mU/mL aprotinin) on ice. For immunoprecipitation, cells were solubilized in the denaturation buffer (1% Triton X-100 lysis buffer containing 0.1% sodium dodecylsulfate [SDS] and 0.5% deoxycholic acid) to dissociate protein complexes. Cell lysates were precleared by centrifugation, and then resultant supernatants were incubated with anti-phosphotyrosine (pTyr) agarose for 1 h at 4 °C, and the immunoprecipitated proteins were eluted by heat treatment at 100 °C for 5 min with 2× sampling buffer.

For the preparation of total cell lysates, cells were washed as described earlier with pre-chilled PBS and lysed with 1% Triton lysis buffer, followed by addition of 3× sampling buffer.

Cell lysates and immunoprecipitated proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and electronically transferred to polyvinylidene difluoride transfer membrane (Millipore, Bedford, MA). After blocking with 5% skim milk in TBST (10 mM Tris, pH 7.4, 150 mM NaCl, and 0.1% Tween 20), the blots were probed with the indicated primary antibodies for 1 h at 4 °C, followed by the appropriate horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature. Finally proteins were visualized by the enhanced chemiluminescence reagent (SuperSignal Western Pico Chemiluminescent Substrate; Pierce, Rockford, IL). The final result was analyzed by NIH Image J software.

*Cell adhesion assay.* Cells were pretreated with cyclosporine A (CsA) or PS-341, followed by the addition of a combination of ATRA and bufalin for 4 d. Ninety-six well polystyrene plates were coated with or without 10 μg/mL human fibronectin (FN, Sigma, St. Louis, MO), incubated at room temperature for 60 min, and blocked with 5% bovine serum albumin (BSA). Cells were harvested and seeded at $5 \times 10^4$/well into the pre-coated plates. For the adhesion assay, non-adherent cells were removed by aspiration with a 23-gauge needle. Cells attached to the plate were measured by MTT assay (Chemicon International, Temecula, CA) according to the manufacturer's instructions. The plate was analyzed using an ELISA reader at 570 nm. At same time, the cells set aside for determination of cell number were also subjected to MTT assay. The final adherent percentage was corrected with total cell number (total cell number of non-adherent cells and adherent cells). The results are shown as mean ± SD of 3–6 experiments.

*Statistical analysis.* In some experiments, statistical significance was determined by Student's *t*-test.

## Results

### *Bufalin enhanced ATRA-induced HL-60 differentiation towards granulocytes/monocytes*

We and others have demonstrated that 5 nM of bufalin clearly enhances ATRA-induced NB4 cell and APL primary cell differentiation [5–7]. To investigate whether the same phenomenon occurs in HL-60 cells, HL-60 cells were treated with 30 nM ATRA, 5 nM bufalin, or the combination of 30 nM ATRA and 5 nM bufalin for 4 d. The expression of CD11b, a marker of HL-60 cell differentiation toward granulocytes/monocytes, was analyzed. Control

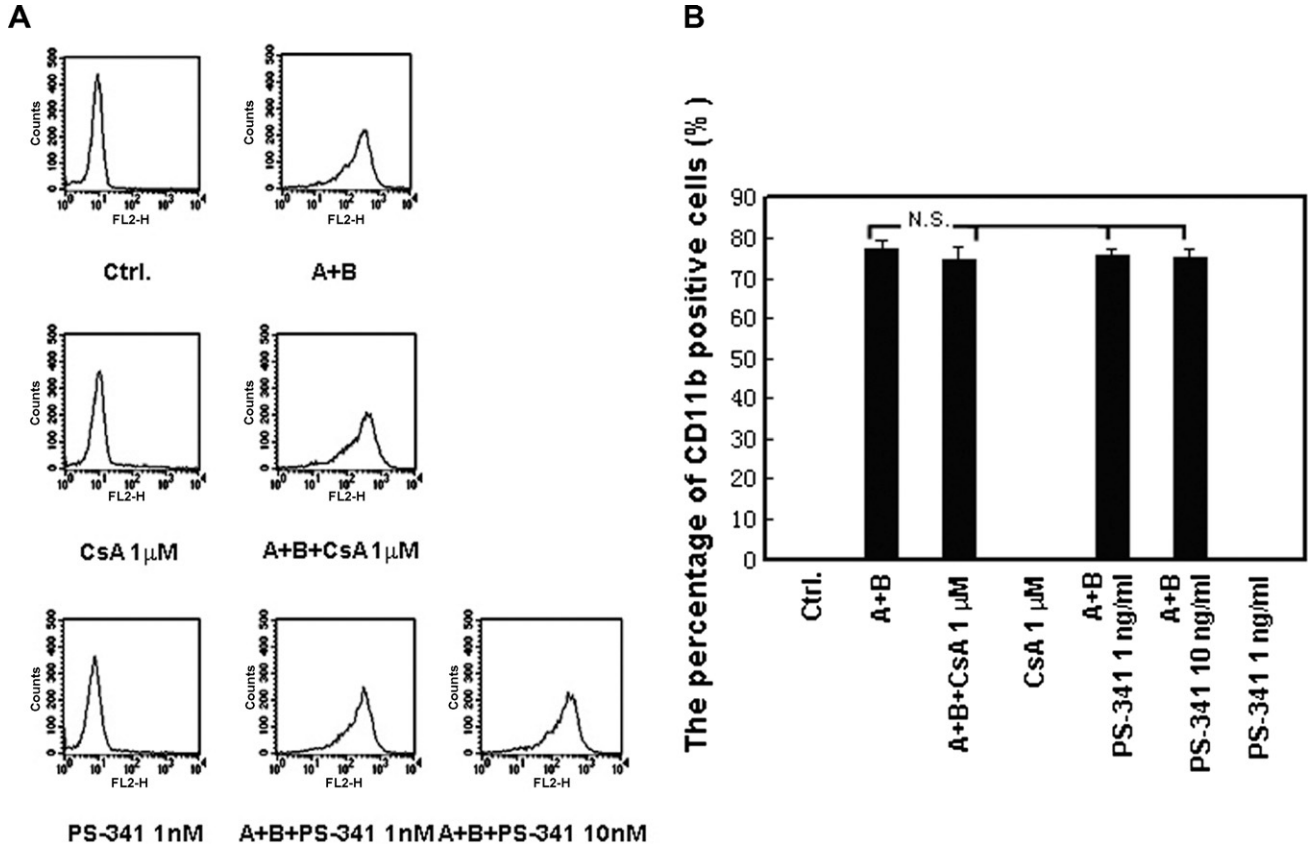


Fig. 2. Effects of CsA and PS-341 on HL-60 cell differentiation induced by ATRA and bufalin. (A) HL-60 cells were pre-treated with CsA (1 μM) or PS-341 (1 or 10 nM), followed by the addition of a combination of 30 nM ATRA and 5 nM bufalin for 4 d. The expression of CD11b was analyzed by FACS. The final results were summarized in the column graphs (B). Student's *t*-test was applied for to compare ATRA and bufalin-treated groups and another tested group, respectively. *NS* indicates no significant differences. *A + B* indicated the combination of ATRA and bufalin.

HL-60 cells and cells exposed to bufalin alone did not express CD11b, and cells exposed to ATRA alone showed relatively low CD11b expression, corresponding to $28.7 \pm 0.32\%$ of total cells (Fig. 1A). Cells exposed to the combination of ATRA and bufalin showed higher CD11b expression corresponding to $77.1 \pm 0.53\%$ of total cells, which is similar to our prior study [6]. The morphological analysis also showed the same differentiating tendency (data not show). These results indicated that bufalin facilitated ATRA-induced differentiation of HL-60 cells, and bufalin might be expected to reverse ATRA drug resistance in APL treatments.

*The expression levels of the Cbl family of ubiquitin ligases were dramatically up-regulated during HL-60 differentiation induced by ATRA and bufalin*

HL-60 cells were treated with different combinations of ATRA and bufalin for 4 d. Treatment of 30 nM ATRA increased the expression of c-Cbl and Cbl-b by 2.38 and 1.41 times over the untreated control cells, respectively. Although 5 nM bufalin alone almost had no effect on c-Cbl and Cbl-b expression, it enhanced ATRA-induced up-regulation of the Cbl family of ubiquitin ligases (Fig. 1B). Further investigation showed that the combination of ATRA and bufalin up-regulated the expression of the Cbl family of ubiquitin ligases in a time-dependent manner. The expression of c-Cbl and Cbl-b was gradually up-regulated on day 2, increased sharply and plateaued on day 4, and constitutively maintained the expression level until day 6 (Fig. 1C). These results showed that differentiation of HL-60 cells was accompanied by an increase in Cbl protein expression.

*PLC-γ2 was tyrosine phosphorylated during ATRA and bufalin-induced HL-60 cell differentiation, and CsA reversed the up-regulation of the Cbl family of ubiquitin ligases*

To analyze the mechanism by which Cbl proteins are up-regulated, the calcium-related signal pathway was tested. PLC-γ2, an enhancer of the calcium mobilization, was tyrosine phosphorylated from day 2 of ATRA/bufalin treatment, reached a peak on day 4, and decreased again on day 6 (Fig. 1D). On the other hand, the up-regulation of Cbl proteins was partially reversed by the pretreatment with CsA, the inhibitor of calcium mobilization (Fig. 1E). These results implied that calcium mobilization might be involved in the up-regulation of the Cbl family of ubiquitin ligases during ATRA and bufalin-induced HL-60 cell differentiation.

*Up-regulation of the Cbl family of ubiquitin ligases did not affect the induced expression of CD11b*

Next, we explored whether the up-regulation of Cbl proteins was relevant to cell differentiation. HL-60 cells were pre-treated with 1 μM CsA for 2 h, followed by the addition of 30 nM ATRA and 5 nM bufalin. Although pretreatment of CsA obviously reversed the up-regulation of c-Cbl and Cbl-b (Fig. 1E), it did not decrease the percentage of CD11b positive cells ($74.69 \pm 3.2\%$) (Fig. 2A) compared to ATRA and bufalin treated cells ($77.36 \pm 2.1\%$). PS-341, the proteasome inhibitor and functional inhibitor of the Cbl family of ubiquitin ligases, did not decrease the expression of CD11b [17,18]. The percentages of CD11b positive cells were $75.77 \pm 1.2\%$ and $75.33 \pm 2.1\%$ of cells exposed to 1 nM PS-341 and 10 nM PS-341 for 4 h, respectively (Fig. 2B). These data suggested that the up-regulation and function of Cbl proteins might not be essential for ATRA and bufalin-induced cell differentiation.

*Adhesion-related kinases Pyk2 and Paxillin were up-regulated during ATRA and bufalin-induced HL-60 cell differentiation*

HL-60 cells were treated with different combinations of 30 nM ATRA and 5 nM bufalin for 4 d. The adhesion-related kinases Pyk2 and Paxillin were up-regulated in a

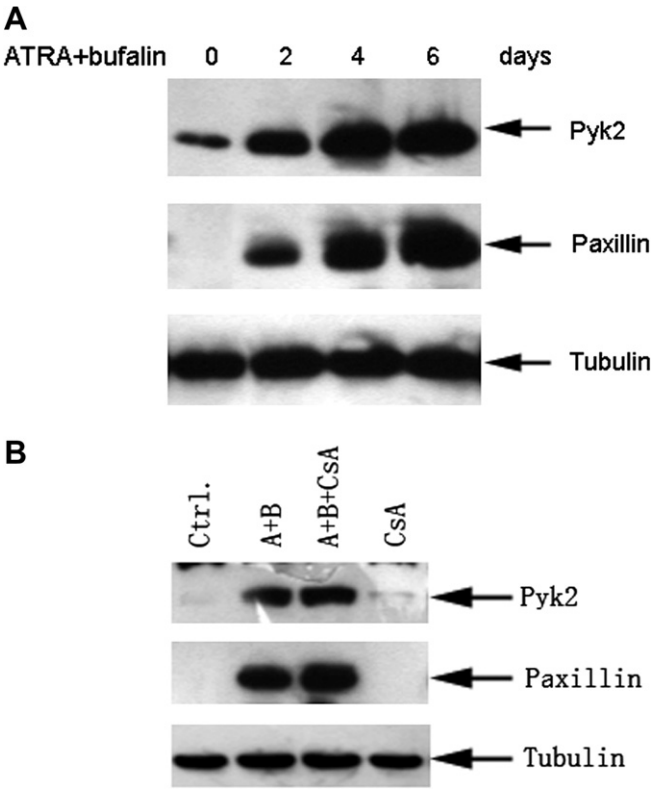


Fig. 3. Effects of ATRA and bufalin on the expression of adhesion-related kinases Pyk2 and Paxillin. (A) HL-60 cells were treated with a combination of 30 nM ATRA and 5 nM bufalin for the indicated number of days. Cell lysates were separated by SDS–PAGE, followed by Western blotting with anti-Pyk2, anti-Paxillin and anti-tubulin mAbs. (B) HL-60 cells were pre-treated with or without CsA (0.1 or 1 μM) or PS-341 (1 or 10 nM), followed by the addition of a combination of 30 nM ATRA and 5 nM bufalin for 4 d. Cell lysates were separated by SDS–PAGE, followed by Western blotting with anti-Pyk2, anti-Paxillin and anti-tubulin mAbs.

time-dependent manner, which paralleled the increase of Cbl proteins. The expression level of Pyk2 reached a plateau on day 4, while the expression level of Paxillin was constitutively increased until day 6 (Fig. 3A). Further investigation showed that 1 μM CsA did not decrease the expression of Pyk2 and Paxillin, unlike the changes in Cbl proteins (Fig. 3B). These results implied that ATRA and bufalin-induced HL-60 cell differentiation was also accompanied by the up-regulation of Pyk2 and Paxillin, which might not depend on the calcium signal pathway.

*The expression of Cbl proteins affected cell adhesion*

Since adherent ability is required for the function of granulocytes/monocytes, we examined the effects of the Cbl family of ubiquitin ligases on cell adhesion. As shown in Fig. 4A, cells treated with ATRA and bufalin increased the percentage of adherent cells to $9.44 \pm 0.8\%$ in the absence of FN, and $11.64 \pm 0.2\%$ in the presence of FN. Pretreatment with CsA partially reversed the induced cell adhesion in a dose-dependent manner. On the other hand,

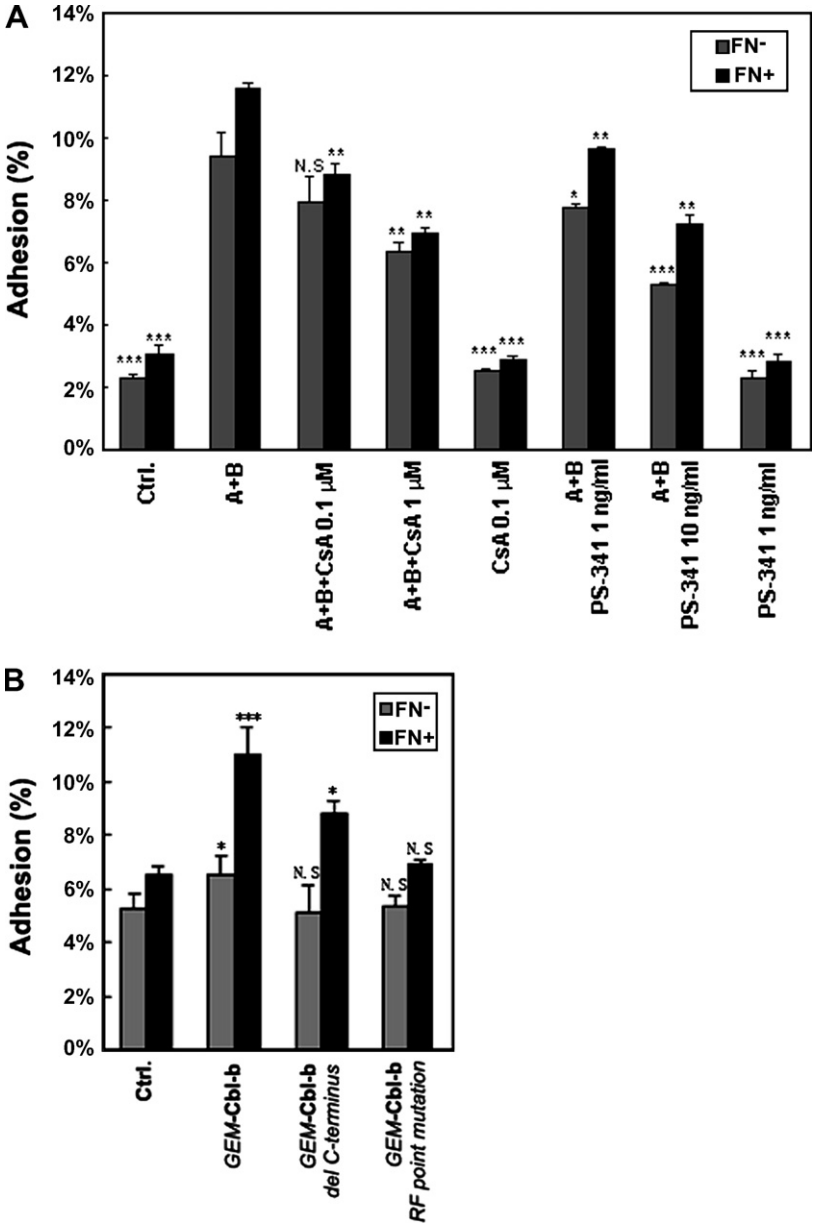


Fig. 4. Effects of the Cbl family of ubiquitin ligases on cells adhesion. (A) Ninety six-well polystyrene plates were treated as described in the Materials and Methods. HL-60 cells were pre-treated with or without CsA (0.1 or 1 μM) or PS-341 (1 or 10 nM), followed by the addition of a combination of 30 nM ATRA and 5 nM bufalin for 4 d. Then cells were harvested and seeded in 96-well dish at a concentration of $5 \times 10^4$/well. Cellular adherent ability was analyzed by MTT assay. $A + B$ indicates the combination of ATRA and bufalin. The Student's $t$-test was applied to compare the ATRA and bufalin-treated group with another test group, respectively. (B) Rat basophilic leukemia-2H3 cells were seeded in 96-well dish at a concentration of $5 \times 10^4$/well, and cultured for 3 h. Cellular adherent ability was analyzed by MTT assay. The Student's $t$-test was applied to compare RBL-2H3 cells and transfected cells. *NS* indicates no significant differences; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

cells pre-treated with PS-341 for 4 h also reversed cell adhesion in a dose-dependent manner. Previous studies established that rat basophilic leukemia-2H3 cell lines over-express different forms of Cbl-b in a lipid raft (more than 50% of total Cbl-b). Lipid rafts were used to further examine the special structural requirements of the Cbl family of ubiquitin ligases on cell adhesion. Those cell lines showed obviously different percentage of adherent cells after 3 h culture. Over-expression of Cbl-b in the lipid raft increased the percentage of adherent cells. However, as shown in Fig. 4B, both RING Finger domain point mutation and C-terminal deletion of Cbl-b partially abolished the increased adhesion. These results indicated that the up-regulated expression of the Cbl family of ubiquitin ligases was involved in cell adhesion, which was mainly dependent on the lipid raft localization, ubiquitin ligase activity and C-terminal region of Cbl-b.

## Discussion

The Cbl family of ubiquitin ligases includes multifunctional proteins in different cell signaling pathways. Jeschke et al. reported that c-Cbl is strongly induced during PMA-induced differentiation toward osteoclast cells [15]. The study by Keane et al. demonstrates that c-Cbl and Cbl-b expression is up-regulated during TPA induced differentiation of HL-60 and U937 cells [16]. But the mechanism of the up-regulation of Cbl proteins has not been explored. In T cells, Cbl proteins expression is up-regulated by the treatment with calcium ionophore, and the inhibitor of calcium mobilization, CsA, reverses this induced expression [10]. The present study showed that the expression of Cbl proteins was increased in a time-dependent manner during ATRA and bufalin-induced HL-60 cell differentiation toward granulocyte/monocyte cells. The calcium mobilization enhancer, PLC-γ2, was tyrosine phosphorylated, which paralleled the inducible expression of Cbl proteins. CsA partially reversed the up-regulation of c-Cbl and Cbl-b, similar to the results observed in T cells [10]. Together with the report that bufalin is a kind of Na/K ATPase inhibitor that is able to lead the calcium influx [19], the present results indicated that ATRA and bufalin-induced up-regulation of the Cbl family of ubiquitin ligases might result from calcium mobilization.

It has been reported that c-Cbl is required for the regulation of the cytoskeleton, and subsequent cell spreading and migration [20,21]. The up-regulation of c-Cbl accompanied by the increase of Pyk2 enhanced the adherence of FLG 29.1 cells to FN [15]. Pyk2 and c-Cbl were found co-localized with actin in axons and growth cones of differentiated PC12 cells, and the formation of growth cone lamellipodia was dependent on intact lipid rafts and phosphatidylinositol 3 (PI-3)-kinase [22]. Moreover, Src-like adaptor protein (SLAP) enhances cell adhesion by facilitating the membrane association of c-Cbl, and the interaction between p85 of PI-3 kinase and c-Cbl was important for cell adhesion and spreading [23,24]. The present study showed that the induced up-regulation of Cbl proteins was accompanied by an increase in Pyk2 and Paxillin, which paralleled the enhanced adhesion of differentiated HL-60 cells. And the abrogated up-regulation of Cbl proteins was accompanied by suppression of differentiated cell adhesion, in concordance with a report showing that the Cbl family of ubiquitin ligases are important modulators of actin cytoskeletal dynamics in diverse biological systems, and facilitate cell spreading and adhesion [23]. The present results also showed that over-expression of Cbl-b in the lipid raft enhanced cell adhesion [25], and RING Finger domain point mutation reversed the enhanced adhesion. Moreover, C-terminal deletion of Cbl-b, which dissociated the interaction between c-Cbl and p85, also partially suppressed the facilitated adhesion of differentiated cells. Together, these data suggest that up-regulated Cbl proteins function in cell adhesion. Furthermore, lipid raft localization, ubiquitin ligase activity, and C-terminus of Cbl proteins were essential for the adhesion. Also, the up-regulation of Pyk2 and Paxillin were also implicated in this regulation.

In conclusion, expression of Cbl ubiquitin ligases was up-regulated during ATRA and bufalin-induced HL-60 cell differentiation. The inducible up-regulation of c-Cbl and Cbl-b affected cell adhesion rather than cell differentiation. The role of the Cbl family of ubiquitin ligases on cell adhesion was dependent on lipid raft localization, ubiquitin ligase activity and the C-terminal structure of Cbl proteins. The adhesion-related kinases Pyk2 and Paxillin may participate in the regulation of adhesion by Cbl proteins. The present study indicated that the Cbl family of ubiquitin ligases may be involved in the regulation of differentiated HL-60 cell adhesion.

## Acknowledgments

We thank Dr. Hirohei Yamamura and Dr. Kiyonao Sada for kindly providing the reagents. We also thank Dr. Kiyonao Sada for the advice on our manuscript. This paper was supported by National Natural Science Foundation of China (No. 30770993, No. 30700807).

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