c-Abl acetylation by histone acetyltransferases regulates its nuclear–cytoplasmic localization

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c-Abl function is strictly dependent on its subcellular localization. Using an *in vitro* approach, we identify c-Abl as a new substrate for p300, CBP (CREB-binding protein) and PCAF (p300/CBP-associated factor) histone acetyltransferases. Remarkably, acetylation markedly alters its subcellular localization. Point mutagenesis indicated that Lys730, located in the second nuclear localization signal, is the main target of p300 activity. It has previously been reported that c-Abl accumulates in the cytoplasm during myogenic differentiation. Here, we show that c-Abl protein is acetylated at early stages of myogenic differentiation. Indeed, acetylation on Lys730 drives c-Abl accumulation in the cytoplasm and promotes differentiation. Thus, Lys730 acetylation is a novel post-translational modification of c-Abl and a novel mechanism for modulating its subcellular localization that contributes to myogenic differentiation.
Keywords: cAbl; p300; acetylation; subcellular localization; myogenic differentiation

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INTRODUCTION

The ubiquitously expressed non-receptor tyrosine kinase c-Abl localizes to the nucleus and to the cytoplasm. In fact, c-Abl has three nuclear localization signals (NLSs) and one nuclear export

signal (NES) that ensure its shuttling between the nucleus and the cytoplasm (Wen *et al*, 1996; Taagepera *et al*, 1998). The output of c-Abl activation is strictly dependent on its subcellular localization. c-Abl activation in the nucleus occurs after DNA damage and also by death receptor stimulation, and participates in the apoptotic response (Yuan *et al*, 1997; Dan *et al*, 1999; Barilà *et al*, 2003). Conversely, c-Abl is activated in the cytoplasm by growth factor receptor stimulation and participates in the transduction of proliferative signals (Plattner *et al*, 1999; Furstoss *et al*, 2002). c-Abl exits from the nucleus and relocalizes to the focal adhesions after cell adhesion (Lewis *et al*, 1996). Conversely, during the apoptotic response, c-Abl accumulates in the nuclear compartment (Barilà *et al*, 2003; Yoshida *et al*, 2005). Both caspases and the 14-3-3 proteins modulate c-Abl accumulation in the cytoplasm and in the nucleus (Barilà *et al*, 2003; Yoshida *et al*, 2005).

Recently, protein acetylation, triggered by histone acetyltransferases (HATs), such as p300/CBP (CREB-binding protein) and PCAF (p300/CBP-associated factor), has been proposed as a new mechanism for modulating subcellular localization (Bannister *et al*, 2000; Soutoglou *et al*, 2000; Spilianakis *et al*, 2000; Madison *et al*, 2002; Bonaldi *et al*, 2003; Gay *et al*, 2003; Santos-Rosa *et al*, 2003). HATs trigger the transfer of an acetyl group from acetyl coenzyme A to the ϵ -amino group of a lysine residue not only on core histones but also on about 40 transcription factors and on more than 30 other proteins (Yang, 2004).

Here, we show that p300, CBP and PCAF may trigger c-Abl protein acetylation. Acetyltransferase activity drives the accumulation of c-Abl in the cytoplasmic compartment. Point mutagenesis identified Lys730 on the human c-Abl1B isoform as the main target of p300 activity. This residue lies in the second NLS of Abl. We propose that acetylation on Lys730 modulates c-Abl localization. Moreover, we could detect endogenous c-Abl acetylation at early stages of myogenic differentiation and show that this event promotes the pro-differentiation activity of c-Abl.

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Fig 1 | p300 activity drives c-Abl accumulation in the cytoplasmic compartment. (A) Abl/Arg^{-/-} double knockout (dKO) fibroblasts were transiently transfected with c-Abl in the presence or absence of p300 or p300DY. c-Abl and p300 cellular localization was shown with specific antibodies. Nuclei were counterstained with Hoechst. (B) The histogram shows the percentage of cells out of transfected cells that show an equal distribution of c-Abl between nucleus and cytoplasm (N=C), a prevalence of c-Abl in the cytoplasm (C>N) or a prevalence of c-Abl in the nucleus (N>C). The data are expressed as the mean $\pm$ s.d. of five independent transfection experiments in which 400 transfected cells were analysed each time.

RESULTS

p300 activity induces c-Abl cytoplasmic localization

To investigate whether HATs might modulate c-Abl subcellular localization, we performed immunofluorescence experiments on fibroblasts derived from Abl/Arg^{-/-} double knockout (dKO) mice that were transiently co-transfected with c-Abl and p300. Transfected c-Abl showed a diffused distribution in the cytoplasm and in the nucleus. Interestingly, p300 co-transfection caused a marked accumulation of c-Abl mainly in the cytoplasmic compartment (Fig 1A). Indeed, 70% of the co-transfected cells accumulated c-Abl in the cytoplasm (Fig 1B). This event requires Abl protein acetylation, as the HAT-deficient mutant p300DY was unable to drive c-Abl relocalization in the cytoplasm (Fig 1).

HATs trigger c-Abl protein acetylation

To assess whether p300 may trigger c-Abl protein acetylation, fibroblasts derived from Abl/Arg^{-/-} dKO mice were co-transfected with c-Abl and p300. Immunoblotting with anti-acetyl lysine

antibodies on immunoprecipitated Abl protein showed that p300 triggers c-Abl acetylation (Fig 2A). Moreover, co-transfection experiments in human embryonic kidney (HEK)293 cells showed that p300, CBP and also PCAF trigger c-Abl acetylation (Fig 2B). Confirming the observation that HAT activity is required to drive c-Abl accumulation in the cytoplasm (Fig 1), overexpression of the HAT-activity-deficient mutant p300DY was unable to acetylate c-Abl (Fig 2C). The acetylation signal on immunoprecipitated c-Abl is efficiently competed by immunoblotting with anti-acetyl-lysine antibodies along with an acetylated lysine peptide (Fig 2D). Remarkably, we could also detect c-Abl by immunoblotting, in the pool of acetylated proteins, after immunoprecipitation with anti-acetyl-lysine antibodies (Fig 2E). Using a mutant of Abl that selectively accumulates in the nucleus (Barilà *et al*, 2003), we were able to show that acetylation occurs mainly in the nucleus (supplementary Fig 1A online). However, acetylated Abl also accumulates in the cytoplasm, confirming that after acetylation, c-Abl changes its subcellular localization (supplementary Fig 1B online).

c-Abl is acetylated on Lys 730 in the second NLS

To explain how acetylation modulates c-Abl localization, we asked whether lysine residues located in the NLS or in the NES sequences may be directly acetylated. Three mutants of NLS 1 (Abl-5KR), NLS 2 (Abl-K730R) and NLS 3 (Abl-K784R) were produced in which the lysine residues were replaced by arginine (Fig 3A). Two lysine residues located amino-terminally to the NES show good conservation with putative acetylation sites (Gu & Roeder, 1997), and were therefore individually mutated (Fig 3A). These constructs were transiently co-transfected with p300 and their acetylation was assayed by immunoblotting on immunoprecipitated Abl proteins. These experiments identified Lys730 of the second NLS as the main target of acetylation by p300 (Fig 3B,C). Interestingly, this lysine is preceded by a serine residue (Fig 3A), matching with the G/SK motif that has been proposed as a good candidate target for acetylation (Bannister *et al*, 2000).

c-Abl is acetylated during myogenic differentiation

It has previously been reported that during myogenic differentiation, c-Abl relocates from the nucleus to the cytoplasm (Puri *et al*, 2002). Interestingly, HATs are upregulated at early stages of myogenic differentiation and modulate gene transcription, therefore promoting differentiation (McKinsey *et al*, 2001; Poleskaya *et al*, 2001). To test the hypothesis that c-Abl could be acetylated during myogenesis, we carried out differentiation experiments using the myogenic C2C12 cell line. To avoid the differentiation stimulus from cell contact inhibition, cells were plated at low density and differentiation was induced after incubation in differentiation medium (DM). Immunoblot experiments on immunoprecipitated endogenous c-Abl protein showed that it is acetylated after 14 h of incubation in DM. Acetylation increases at 16 h and is downregulated at 18 and 24 h (Fig 4A). Importantly, immunofluorescence experiments allowed us to show that acetylation is rapidly followed by endogenous c-Abl accumulation in the cytoplasmic compartment. Indeed, already at 16 h incubation in DM, c-Abl begins to accumulate in the cytoplasm, where it remains at later time points (Fig 4B). We estimated that during differentiation the percentage of cells showing c-Abl protein accumulation in the cytoplasmic compartment increases

Fig 2 | p300, CBP and PCAF trigger c-Abl acetylation. (A) c-Abl was immunoprecipitated using specific antibodies from cell lysates obtained from Abl/Arg^{-/-} double knockout (dKO) cells transiently co-transfected with c-Abl in the presence of p300, and acetylation was shown by immunoblotting. (B,C) c-Abl was transfected in human embryonic kidney (HEK)293 cells in the presence of the indicated constructs and immunoprecipitated with specific antibodies. Acetylation was shown as described in (A). The arrows point to acetylated Abl protein. (D) c-Abl was transfected in HEK293 cells in the presence of the indicated constructs and immunoprecipitated with specific antibodies. Immunoblotting with anti-acetyl-lysine was carried out in the presence of increasing amounts of histone H4 peptide (Tetra AcLys), as competitive peptide. (E) c-Abl was transfected in HEK293 cells in the presence of the indicated constructs, immunoprecipitated with anti-acetyl-lysine antibodies and then detected by immunoblotting with specific antibodies. CBP, CREB-binding protein; HA, haemagglutinin; IP, immunoprecipitation; PCAF, p300/CBP-associated factor; WB, western blot.

about sixfold (6% in growth medium (GM) and 38% after 24 h of incubation in DM; Fig 4B). This finding was further supported by immunoblotting on cell fractionation experiments, in which the cytoplasmic fraction of Abl slightly increases during differentiation, whereas the nuclear fraction slightly decreases (supplementary Fig S3 online).

Acetylation on Lys 730 drives c-Abl in the cytoplasm

Immunofluorescence experiments on transfected C2C12 cells showed that p300 enhances about twofold the number of cells in which c-Abl is mainly cytoplasmic (Fig 4E). In accordance with this observation, p300 drives the accumulation in the cytoplasm of Abl-K784R, Abl-5KR, AblK1099R and Abl-K1108R, confirming that these lysines are not responsible for p300-induced c-Abl relocalization (supplementary Fig S4A,B online). Abl-K730R mutant is slightly more cytoplasmic than c-Abl, probably because the mutation affects the function of the NLS. Interestingly, p300 completely failed to increase its accumulation further in the cytoplasm, suggesting that acetylation on Lys730 is required to relocalize c-Abl (Fig 4D).

Lys 730 acetylation promotes myogenic differentiation

To investigate whether c-Abl activity and localization could modulate myogenic differentiation, C2C12 cells plated at low

density were transiently transfected with several Abl constructs. These conditions allow a detailed analysis of the kinetic of differentiation. After 24 h from transfection, cells were placed in DM and differentiation was monitored. In this condition, C2C12 cells express some proteins typical of differentiated muscular cells, such as myogenin (supplementary Fig S5B online) and later on myosin heavy chain (MyHC; Fig 4F; supplementary Fig S5A,D online). However, the low density does not allow the formation of multinucleated myotubes. Differentiated cells were identified by MyHC immunoreaction. The expression of c-Abl or its cytoplasmic mutant form, Abl-NLS⁻, significantly increased the rate of differentiation at 24 h (Fig 4F). Interestingly, at 36 h, Abl expression can still accelerate the rate of differentiation; however, at later times, when β-Gal-transfected control cells are already highly differentiated, this effect is lost (supplementary Fig S5A online). Consistent with the latter results, no difference was detected in the late event of multinucleated myotube formation, when similar experiments were performed with C2C12 cells plated at high density (data not shown). In contrast, neither the nuclear form of Abl, Abl-NES⁻, nor the kinase-defective forms, Abl-Kin⁻ and Abl-NLS-Kin⁻, were able to increase differentiation (Fig 4F), indicating that the kinase activity and the cytoplasmic localization of c-Abl contribute to myogenic differentiation. In addition, the non-acetylatable Abl-K730R mutant lost the ability

Fig 3 | p300 drives c-Abl acetylation mainly on Lys 730. (A) Schematic representation of c-Abl constructs. (B,C) Human embryonic kidney 293 cells were co-transfected with several Abl constructs in the presence or absence of p300 and acetylation was assayed after immunoprecipitation, as described in Fig 2. IP, immunoprecipitation; ut, untransfected; WB, western blot.

to accelerate differentiation, indicating that Lys 730 acetylation contributes to the myogenic properties of c-Abl, possibly triggering its cytoplasmic localization.

DISCUSSION

c-Abl tyrosine kinase has been shown to exert different functions depending on its subcellular localization (reviewed by Pendergast, 2002; Wang, 2005).

Lysine N^ε-acetylation has been proposed to modulate the subcellular localization of several proteins (reviewed by Yang, 2004). Lysines are central components of NLSs (reviewed by Poon & Jans, 2005), as their positive charge mediates the interaction with importins (Conti *et al*, 1998). Indeed, acetylation has been shown to target lysine residues that are located in the NLSs of several proteins. This event may directly modulate NLS function (Bonaldi *et al*, 2003) and may disrupt its association with the import machinery (Madison *et al*, 2002). Alternatively, acetylation inside NLSs, rather than affecting the NLS function itself, may trigger a conformational change that impairs the function of the NES, promoting protein accumulation in the nucleus (Soutoglou *et al*, 2000; Spilianakis *et al*, 2000).

We were able to show that p300, CBP and PCAF can trigger c-Abl protein acetylation mainly on Lys 730, located in the second NLS. This residue is also conserved in the sequence of the Abl-related gene product, Arg, suggesting that this event may have a more general role throughout Abl family proteins. Structural data suggest that this region is not required for the regulation of c-Abl

kinase activity (Barilà & Superti-Furga, 1998; Pluk *et al*, 2002; Hantschel *et al*, 2003; Nagar *et al*, 2003). Indeed, acetylation does not modulate c-Abl catalytic activity (supplementary Fig S2 online). However, in agreement with previous reports on the acetylation of lysine residues located in NLSs (Madison *et al*, 2002; Bonaldi *et al*, 2003), p300 modulates c-Abl subcellular localization and forces protein accumulation in the cytoplasmic compartment. This requires p300 acetyltransferase activity as well as the presence of Lys 730 on the Abl protein. We can speculate that, as reported for HMGB1 and E1A (Madison *et al*, 2002; Bonaldi *et al*, 2003), acetylation negatively affects the function of the second NLS of c-Abl, impairing exported acetyl-Abl's ability to re-enter the nuclear compartment.

It has recently been shown that during muscle differentiation, c-Abl protein accumulates in the cytoplasm (Puri *et al*, 2002). Interestingly, HATs modulate differentiation of muscle cells (McKinsey *et al*, 2001 and references therein; Poleskaya *et al*, 2001) and inhibitors of nuclear deacetylases can promote skeletal myogenesis (Iezzi *et al*, 2002). Here, we were able to show that endogenous c-Abl is acetylated and accumulated in the cytoplasm at early stages of myogenic differentiation. In this system, acetylation peaks at 16 h and is downregulated at later time points (Fig 4A). In parallel, starting from 16 h, c-Abl protein stably accumulates in the cytoplasm (Fig 4B; supplementary Fig S3 online), suggesting that this modification may initiate c-Abl relocation, which may be further stabilized by alternative mechanisms previously reported to promote c-Abl accumulation

Fig 4 | c-Abl acetylation on Lys 730 promotes myogenic differentiation. (A) C2C12 cells were either grown in growth medium (GM) or incubated for different durations in differentiation medium (DM). A 7 mg portion of protein extracts at different stages of differentiation was immunoprecipitated with specific anti-Abl antibodies and acetylation was shown using specific anti-acetyl-lysine antibodies. One-tenth of the immunoprecipitated material was immunoblotted with anti-Abl antibodies as a control. The arrow indicates the acetylated Abl. (B) C2C12 cells were either grown in GM or incubated for different durations in DM. Endogenous c-Abl was detected by immunofluorescence with specific antibodies. Nuclei were counterstained with Hoechst. (C) Quantification of the immunofluorescence experiment shown in (B), counting the percentage of transfected cells in which c-Abl is either diffused or predominantly cytoplasmic. The data are expressed as the mean $\pm$ s.d. of three independent immunofluorescence experiments in which 150 cells were analysed each time. (D) C2C12 cells were transiently transfected and c-Abl subcellular localization was shown by immunofluorescence and (E) quantified as in Fig 1. (F) C2C12 cells were transiently transfected with several constructs of Abl or with β -Gal as a control. At 24 h after transfection, cells were incubated in DM for a further 24 h. Differentiation was assayed by immunofluorescence, counting the percentage of transfected cells that were positive for myosin heavy chain (MyHC). The data are expressed as the mean $\pm$ s.d. of three independent transfection experiments in which 200 transfected cells were analysed each time. IP, immunoprecipitation; WB, western blot.

in the cytoplasm, such as c-Abl interaction with cytoskeletal components or with 14-3-3 proteins (Yoshida *et al*, 2005).

We report for the first time that c-Abl may promote the myoblast–myocyte transition in C2C12 cells. This effect is strictly dependent on c-Abl tyrosine kinase activity and on its ability to accumulate in the cytoplasm. In addition, mutation of Lys 730 severely affects the ability of c-Abl to promote differentiation, suggesting that c-Abl acetylation may have a role in myogenesis. Importantly, c-Abl activity promotes MyHC, but not myogenin, expression at early stages of differentiation, whereas it does not modulate late differentiation. Altogether, these experiments allow us to describe acetylation as a new molecular mechanism to drive c-Abl accumulation in the cytoplasm. Moreover, we identify a novel pro-differentiation function of c-Abl in myogenesis. We speculate that, in this context, acetylation may contribute to the accumulation of c-Abl in the cytoplasmic compartment, where it promotes differentiation.

METHODS

DNA constructs. pCDNA3-c-Abl-5KR, pCDNA3-c-Abl-K730R, pCDNA3-c-Abl-K784R, pCDNA-c-Abl-K1099R and pCDNA-c-Abl-K1108R were generated using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using pCDNA3-c-Abl as template. All the other Abl constructs were described previously (Barilà *et al*, 2003). pCMV-Myc-p300 and pCMV-Myc-p300-DY were provided by T.P. Yao (Duke University, Durham, NC, USA). pCDNA3-HA-CBP and pCI-FLAG-P/CAF were provided by Antonio Costanzo ('Tor Vergata' University, Rome, Italy).

Antibodies. The following antibodies were used: anti-Abl (Ab3; Oncogene; Calbiochem, Merck Darmstadt, Germany), anti-acetyl-lysine (used for immunoblotting; Upstate Cell Signalling Solutions, Charlottesville, VA, USA), anti-acetyl-lysine (used for immunoprecipitation; Cell Signalling, Beverly, MA, USA), anti-Myc (A14; Santa Cruz, Santa Cruz, CA, USA), anti-Flag (M2; Sigma, St Louis, MO, USA), anti-HA (Roche, Indianapolis, IN, USA), anti-p300 (N15; Santa Cruz), anti-phosphotyrosine (4G10; UBI), anti-Jun (kindly provided by G. Superti-Furga) and anti-tubulin (Sigma).

The following antibodies were used for immunofluorescence: anti-Abl (8E9; Pharmingen, San Diego, CA, USA), anti-Myc (A14; Santa Cruz), anti-MyHC (kindly provided by G. Salvatori) and anti- β -Gal (Roche).

Cell culture and transfections. HEK293 cells and Abl/Arg^{−/−} dKO fibroblasts (kindly provided by A. Koleske) were cultured and transfected as described previously (Barilà *et al*, 2003). C2C12 myoblast cells were propagated in GM (DMEM supplemented with 10% FCS), and differentiation was induced by DM (DMEM serum free plus insulin 10 μ g/ml and transferrin 5 μ g/ml). C2C12 myoblasts were transfected with Lipofectamine and Plus-Reagent (Invitrogen, Carlsbad, CA, USA) or with Eugene (Roche).

Protein extracts, immunoblotting, immunoprecipitation and immunofluorescence. Total, nuclear and cytoplasmic cell extracts were prepared as described previously (Barilà *et al*, 2003). For immunoblotting, 100 μ g of protein extract was separated by SDS–polyacrylamide gel electrophoresis. Peptide competition assay was performed by incubating western blots with anti-acetyl-lysine antibody in the presence of histone H4 (Tetra AcLys) peptide (UBI).

Immunoprecipitations were carried out from 4 mg of protein extracts.

For immunofluorescence, cells were treated as described previously (Barilà *et al*, 2003). Immunofluorescence experiments on endogenous Abl were performed as described previously (Puri *et al*, 2002).

Immunofluorescence images were captured using confocal microscopy (Nikon, PCM2000).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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