Introduction: We previously reported that p204 acted as a transcriptional coactivator of Cbfa1 and enhanced osteogenesis (1) and that pRb is an essential linker between p204 and Cbfa1 (2). Id2 protein that binds to p204 functions as a global regulator of gene expression during cell growth and differentiation. The purpose of this study is to elucidate the molecular events underlying Cbfa1, p204 and Id2 protein interaction network in osteogenesis.

Materials and Methods: Construction of adenoviruses expressing Id2, p204 and p204AS; Alkaline Phosphatase and Osteocalcin Assays; Coimmunoprecipitation Assay; Purification of GST fusion proteins; Cytoplasmic and nuclear fractionation and Western blotting analyses; Assay of the acceleration of the degradation of Id2 by p204 in vivo.

Results: Id2 associates with Cbfa1 and inhibits Cbfa1-activated osteogenic differentiation: we first determined whether Id2 associates with Cbfa1. Cell lysates were prepared from C2C12 cells treated with BMP-2 for 8 hours and Co-IP assay was performed. As shown in Fig. 1A, a specific Id2 band was immunoprecipitated by anti-Id2 and anti-Cbfa1 antibody, but not by control IgG, demonstrating that Id2 associates with Cbfa1 in the BMP-2-triggered osteogenesis. We next examined whether Id2 affects the Cbfa1 activity (Fig. 1B). Infection of C2C12 cells with the adeno virus encoding Cbfa1 (Ad-Cbfa1) led to a high ALP activity. This Cbfa1-dependent induction of ALP was inhibited by coinfection with Ad-Id2 in a dosage-dependent manner. Id2 also inhibited Cbfa1-mediated osteocalcin production (not shown). These data suggest Id2 protein is a potent inhibitor of Cbfa1-mediated gene activation in osteogenesis.

p204 disturbs Id2/Cbfa1 complex and overcomes the inhibition of Cbfa1-mediated osteogenic differentiation by Id2: Our previous finding that p204 binds to cbfa1 and enhances its activity together with the facts that Id proteins also bind to Cbfa1 and inhibit Cbfa1-dependent gene activation and osteogenesis, led us to investigate whether p204 prevented the binding of Id2 to Cbfa1. As revealed in Fig. 1C, anti-Flag antibody efficiently immunoprecipitated Cbfa1 from the cell extracts of C2C12 cells transfected with Cbfa1 and Id2-Flag expression plasmids, whereas addition of purified GST-p204 in the cell lysates abolished this complex. Fig. 1D reveals that p204 was capable of overcoming the inhibition of Cbfa1 by Id proteins. Cbfa1-activated ALP activity was reduced when Id2 protein was co-expressed and this repression of Id2 on Cbfa1 action was overcome by p204. Id2 translocates from the nucleus to the cytoplasm in C2C12 cells undergoing osteogenic differentiation induced by BMP-2, and p204 accelerates this translocation: In order to better understand the localization of Id proteins following BMP-2 stimulation, we performed Western blot analysis of nuclear and cytoplasmic fractions from cell lysates (Fig. 2A). Induced Id2 by BMP-2 first appeared in the nucleus and later translocated to the cytoplasm (top panel). We next examined whether p204 mediated the cytoplasmic translocation of Id2 during osteogenesis. As shown in Fig. 2A (bottom panel), the cytoplasmic translocation of Id2 protein was accelerated by Ad-p204 infection in the BMP-treated C2C12 cells. p204 accelerates the degradation of Id2 by ubiquitin-proteasome pathway: Overexpression of p204 enhanced, whereas low level of p204 by antisense approach reduced, the degradation of Id2 in the course of osteogenesis of C2C12 cells induced by BMP-2 (Fig. 2B). In addition, our very recent data demonstrated that the acceleration by p204 of the degradation of Id2 by proteasomes was dependent on ubiquitination.

Discussion: This study provides evidence showing that Id2 protein associates with Cbfa1 and inhibits its osteogenic action and that p204 overcomes this inhibition in C2C12 cells undergoing osteogenic differentiation. Cbfa1, p204 and Id proteins form a regulatory circuit and act in concert to regulate osteoblast differentiation (Fig. 3).


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