BOC, an Ig superfamily member, associates with CDO to positively regulate myogenic differentiation

Jong-Sun Kang, Philip J. Mulieri, Yulan Hu, Lavinia Taliana1 and Robert S. Krauss2

Department of Biochemistry and Molecular Biology and 1Department of Ophthalmology, Mount Sinai School of Medicine, New York, NY 10029, USA
2Corresponding author
e-mail: Robert.Krauss@mssm.edu

CDO is a cell surface receptor-like protein that positively regulates myogenic differentiation. Reported here is the identification of BOC, which, with CDO, defines a newly recognized subfamily within the immunoglobulin superfamily. cdo and boc are co-expressed in muscle precursors in the developing mouse embryo. Like CDO, BOC accelerates differentiation of cultured myoblast cell lines and participates in a positive feedback loop with the myogenic transcription factor, MyoD. CDO and BOC form complexes in a cis fashion via association of both their ectodomains and their intracellular domains. A soluble fusion protein that contains the entire BOC ectodomain functions similarly to full-length BOC to promote myogenic differentiation, indicating that the intracellular region is dispensable for its activity in this system. Furthermore, a dominant-negative form of CDO inhibits the pro-myogenic effects of soluble BOC, suggesting that BOC is dependent on CDO for its activity. CDO and BOC are proposed to be components of a receptor complex that mediates some of the cell–cell interactions between muscle precursors that are required for myogenesis.

Keywords: cell differentiation/Ig superfamily/MyoD/myogenesis

Introduction

In vertebrates, skeletal muscles of the body arise from somites, spheres of columnar epithelial cells that develop from unsegmented paraxial mesoderm (Tajbakhsh and Buckingham, 2000). Muscle precursor cells are derived from the dorsal epithelial portion of the maturing somite, the dermomyotome, and subsequently will either form the myotome, a set of differentiated muscle cells that underlies the dermomyotome, or migrate to form more distal muscles. The generation of myogenic cells in somites is regulated by both positive and negative signals derived from neighboring tissues, including the neural tube, notochord, surface ectoderm and lateral plate mesoderm (Borycki and Emerson, 2000). However, determination and differentiation of cells in the vertebrate skeletal muscle lineage also require specific cell–cell interactions between muscle precursors, a phenomenon termed the ‘community effect’ (Gurdon et al., 1993; Cosu et al., 1995). Moreover, cell aggregation is a pre-condition for differentiation of certain myogenic cell lines, and high cell density promotes differentiation of C2C12 and other well-studied myoblast cell lines (Skerjanc et al., 1994; Redfield et al., 1997). The cell surface proteins that mediate such effects are not known, although various cadherins, immunoglobulin (Ig) superfamily members and sialomucins have been proposed to play a role (Dickson et al., 1990; Holt et al., 1994; Zeschnigk et al., 1995; Redfield et al., 1997; Kang et al., 1998; Lee et al., 2001).

Development of the skeletal muscle lineage is critically dependent on a positive feedback network of transcription factors, including the four myogenic basic helix–loop–helix (bHLH) factors (MyoD, Myf-5, myogenin and MRF4) and members of the MEF-2 family (Yun and Wold, 1996; Black and Olson, 1998; Arnold and Braun, 2000). The components of this core network function individually and together to maintain their own expression; during differentiation, they orchestrate exit from the cell cycle and expression of lineage-specific genes. Furthermore, myogenic bHLH and MEF-2 factors are targets of signal transduction pathways that influence myogenesis (Puri and Sartorelli, 2000).

We are interested in cell contact-mediated regulation of myogenesis and are studying the role of CDO, a cell surface receptor-like protein, in this process. CDO contains five Ig repeats followed by three fibronectin type III (FNIII) repeats in its extracellular domain, a single-pass transmembrane domain and a 270 amino acid intracellular region that does not resemble other known proteins (Kang et al., 1997). The five Ig + three FNIII repeat arrangement of CDO’s ectodomain is shared by the Robo proteins, an evolutionarily conserved subfamily of axon guidance receptors (Simpson et al., 2000).

Several lines of evidence indicate that CDO positively regulates myogenesis. cdo mRNA is expressed at high levels in newly arising somites, dermomyotomes and myotomes, as well as in myoblasts and early muscle mass of the trunk and limb bud (Kang et al., 1998; Mulieri et al., 2000). Overexpression of CDO in C2C12 and other myoblast cell lines accelerates differentiation, while expression of a secreted, soluble form of the CDO extracellular region functions as a dominant-negative factor to inhibit this process (Kang et al., 1998). Transformation of C2C12 cells with the ras oncoprotein results in down-regulation of MyoD and cdo expression, and a blockade to differentiation (Kang et al., 1998). Forced re-expression of CDO in such cells leads to induction of endogenous MyoD and, conversely, forced re-expression of MyoD leads to induction of endogenous cdo (Kang et al., 1998). Re-expression of either CDO or MyoD in ras-transformed C2C12 cells produces an identical, highly specific phenotype: reactivation of the differenti-
ation program without alteration of other parameters of transformation, such as anchorage-independent growth (Lassar et al., 1989; Kang et al., 1998). These and additional results suggest that a positive feedback loop exists between CDO and myogenic bHLH factors, and that CDO may be an integral part of the myogenic regulatory network. Thus, CDO may be the first identified component of an extended positive feedback network that regulates, and is in turn regulated by, the core nuclear network of MyoD family members and MEF-2 proteins.

We report here the identification and characterization of BOC (brother of CDO), another member of the Ig/FNIII repeat family of receptor-like proteins. BOC has a four Ig plus three FNIII structure in its ectodomain, but is much more closely related to CDO at the amino acid level than are the Robo axon guidance receptors, despite the latter sharing a 5 + 3 structure with CDO. The developmental expression patterns of \textit{boc} and \textit{cdo} overlap significantly, and BOC displays properties much like CDO \textit{in vitro}. BOC and CDO form complexes that act to promote differentiation of myoblasts. Taken together, the data indicate that BOC is an additional participant in the extended positive feedback loop postulated to exist for CDO and myogenic bHLH factors, and that CDO and BOC may be components of a receptor complex that mediates some of the effects of cell contact between muscle precursors during myogenesis.

Results

\textbf{Identification of BOC}

\textit{boc} was identified initially by low stringency screening of a human fetal brain cDNA library with a rat \textit{cdo} cDNA probe. Comparison of this initial cDNA clone with expressed sequence tag (EST) databases and screening of human, mouse and \textit{Xenopus} cDNA libraries permitted derivation of full-length \textit{boc} open reading frames (ORFs) for all three species. Human \textit{boc} encodes a 1113 amino acid protein comprised of an extracellular region of four Ig repeats followed by three FNIII repeats, a single-pass transmembrane domain and a 238 amino acid intracellular region. The amino acid identity between human BOC and CDO in their individual extracellular domains ranges from 38\% (the fourth Ig repeat) to 80\% (the third FNIII repeat) (Figure 1A). The second and third FNIII repeats are the most closely related domains between BOC and CDO, as well as between the various CDO and BOC orthologs (Kang et al., 1997; Figure 1A). In contrast, BOC’s intracellular region is not related to that of CDO or to other proteins in the databases; furthermore, motifs that might serve as binding sites for adaptor proteins (e.g. proline-rich stretches) are not obvious in the BOC intracellular region sequence (data not shown).

Examination of the complete \textit{Drosophila} genome sequence revealed two related genes predicted to encode
proteins that share high amino acid identity with CDO and BOC in their extracellular regions. These genes (CG13756 and CG9211) have a four Ig + two FNIII ectodomain topography and, as is seen with CDO and BOC themselves, the domains most closely related to those of CDO and BOC are the membrane-proximal FNIII repeats (Figure 1A). The intracellular regions of CG13756 and CG9211, however, are not obviously related to those of CDO or BOC (data not shown). A different 4 + 2 protein predicted by the Drosophila genome sequence (GH11322) is more closely related to the DCC subfamily, and the Caenorhabditis elegans protein most closely related to CDO and BOC is SAX-3, a member of the Robo subfamily. A phylogenetic tree comprised of CDO and BOC orthologs and their closest relatives in the databases indicates that CDO, BOC, CG13756 and CG9211 form a distinct subgroup of the Ig/FNIII family, distinguishable from the Robo receptors and other family members (Figure 1B).
Expression of boc

Expression of boc during murine embryonic development was assessed by whole-mount and thin section in situ hybridization. Strong expression is observed in the dorsal neural tube and somites (Figure 2A). During skeletal muscle development, shown in Figure 2A on E10.5, boc is expressed at high levels in dermomyotomes and at significantly lower levels in myotomes, the mature central portions of which appear devoid of signal (Figure 2A, b–e). Comparison of signals obtained with probes for boc, Pax3 (a dermomyotome-specific marker) and myogenin (a myotome-specific marker) are consistent with this conclusion and suggest that boc levels are highest in the dorsal lip of the dermomyotome (Figure 2A, f–h). Limb buds also display robust expression of boc on E10.5 (Figure 2A, a). Thus, the developmental expression patterns of boc and cdo overlap significantly (Mulieri et al., 2000, 2002).

Analysis of boc mRNA expression in C2C12 myoblasts revealed an ~5 kb transcript in both proliferating and differentiated C2C12 cells; levels of this transcript decreased over a 4 day differentiation period (Figure 2B). Similarly to cdo and MyoD, boc mRNA levels were sharply reduced in Ras-transformed C2C12 cells cultured under both growth and differentiation conditions (Figure 2C). Interestingly, forced re-expression of MyoD in C2C12/Ras cells, which overrides the Ras-induced block to differentiation (Lassar et al., 1989; Kang et al., 1998), led to restoration of boc expression (Figure 2C). These data closely resemble those obtained for cdo (Kang et al., 1998) and suggest that, like cdo, boc may be a component of the myogenic program.

Expression of boc in adult tissues was analyzed by northern blotting of human poly(A)+ RNA (Figure 2D). boc transcripts are detected in most tissues, most abundantly in skeletal muscle and small intestine. The expression of boc in adult tissues contrasts with that of cdo, which is barely detectable in adult rat or human tissues (Kang et al., 1997; and data not shown).
**BOC positively regulates myogenic differentiation**

To assess the role of BOC in myogenic differentiation, the C2C12 and F3 myoblast cell lines were engineered, via retroviral transduction, to express a human *boc* CDNA that incorporated a flag epitope tag at the C-terminus of the protein [BOC(flag); Figure 3A]. The lower molecular weight band specifically produced by the BOC(flag)-expressing cells seen in Figure 3A and later figures resulted from incomplete N-linked glycosylation of BOC (data not shown).

Overproduction of BOC had no effect on the morphology of C2C12 or F3 cells or on their ability to proliferate in growth medium (GM; data not shown). However, when challenged to differentiate, C2C12 and F3 cells that overexpressed BOC displayed accelerated production of the differentiation markers myogenin, myosin heavy chain (MHC) and troponin T (TnT) relative to control infectants; in contrast, levels of MyoD were unchanged (Figure 3B). Furthermore, the BOC(flag)-expressing cells formed larger myotubes that contained more nuclei than did the control cells [Figure 3C; 64% versus 25% of nuclei in myotubes for BOC(flag)-expressing versus control C2C12 cells, and 30% versus 13% of nuclei in myotubes for BOC(flag)-expressing versus control F3 cells (average values from four experiments)]. Thus, as is seen with CDO, overexpression of BOC results in precocious differentiation of myoblasts as assessed by both biochemical and morphological criteria, and this occurs without alteration in the level of MyoD.

Oncogenic Ras down-regulates expression of MyoD and CDO in C2C12 cells, blocking differentiation and inducing neoplastic transformation; forced re-expression of either protein reactivates the myogenic program in these cells, without altering their ability to form colonies in soft agar (Lassar et al., 1989; Kang et al., 1998). Because *boc* mRNA expression is also down-regulated by Ras (Figure 2C), the ability of BOC to reactivate myogenesis was examined. C2C12/Ras cells infected with either control or BOC(flag) retroviruses (Figure 4A) were cultured in differentiation medium (DM) and analyzed biochemically and morphologically for signs of differentiation, relative to a double-vector control line. C2C12/Ras cells infected with the control virus expressed very low levels of muscle-specific proteins (Figure 4B). In contrast, the BOC(flag) virus infectants expressed MyoD and myogenin in GM and induced expression of MHC and TnT in DM, albeit at reduced levels relative to the double-vector control (Figure 4B). Furthermore, the BOC(flag)-
expressing cells reacquired the ability to form multinucleate myotubes in DM (Figure 4C). However, these cells still formed colonies in soft agar with a frequency similar to that of C2C12/Ras cells infected with the control virus (~15%). Therefore, similarly to previous observations with CDO and MyoD, re-expression of BOC is sufficient to restore a functional differentiation program to Ras-transformed C2C12 myoblasts.

**BOC and CDO form complexes**

cdo and boc are co-expressed during development and in C2C12 myoblasts, and they display nearly identical phenotypes when ectopically expressed in control and Ras-transformed C2C12 cell derivatives (Figures 2-4). These data suggested that CDO and BOC may function together as components of a complex. To test whether CDO and BOC can bind to each other, lysates from C2C12 cells infected with either control or BOC(FLAG) retroviruses were immunoprecipitated with antibodies to the flag epitope and then immunoblotted with antibodies to CDO. As can be seen in Figure 5A, the flag antibody efficiently co-immunoprecipitated endogenous CDO in lysates from the BOC(FLAG)-expressing cells, but not those from the vector control cells.

To investigate complex formation between CDO and BOC in more detail, transient transfections in 293T cells were performed. A fixed amount of BOC(FLAG) expression vector was co-transfected with increasing amounts of a CDO expression vector. As was seen in C2C12 lysates, flag antibodies co-immunoprecipitated CDO (Figure 5B, left panel). Additionally, increasing the amount of transfected cdo vector increased the amount of BOC(FLAG) protein in the immunoprecipitates. Conversely, increasing the amount of transfected boc vector also increased the amount of CDO produced from a fixed amount of vector (data not shown), suggesting that complex formation might stabilize these proteins. Formation of a complex between CDO and BOC was also observed when the precipitations were performed with antibodies to CDO and the precipitates were immunoblotted with antibodies to the flag epitope (Figure 5B, middle panel). As a control for specificity, the ability of CDO or BOC to form complexes with neural cell adhesion molecule (NCAM) was tested. NCAM has a five Ig + two FNIII topography in its ectodomain and, like CDO and BOC, is expressed in developing muscle and C2C12 cells (Moore et al., 1987; Lyons et al., 1992). As can be seen in Figure 5B (right panels), two different isoforms of NCAM (180 and 140 kDa) failed to associate with CDO or BOC in transiently transfected cells, indicating that the association between CDO and BOC is specific.

To determine what regions of CDO and BOC might mediate their interaction, the ability of two deletion mutants of CDO to bind to BOC(FLAG) was assessed. The deletion mutants are CDO(ΔICR1), a construct in which 95% of CDO’s cytoplasmic region was removed, and CDO(TMtrna), a construct in which the CDO signal sequence was linked directly to its transmembrane and cytoplasmic regions. Expression vectors for these deletion mutants were co-transfected individually with the BOC(FLAG) vector. Cell lysates were precipitated with anti-flag antibodies and then blotted with antibodies to either the extracellular or intracellular regions of CDO. As shown in Figure 5C, both CDO(ΔICR1) and CDO(TMtrna) co-precipitated with BOC-FLAG. The association of both these deletion mutants of CDO with BOC suggests that their interaction occurs in cis (in the plane of the same cell membrane), rather than in trans (between apposing cell membranes). Consistent with this notion, when 293T transfecants that expressed only CDO were co-cultured with transfecants that expressed only BOC, immunoprecipitation of either protein failed to co-precipitate the other, an interaction that should be observed if binding occurred in trans (data not shown).

The formation of cis complexes between CDO and BOC suggests that their respective extracellular and/or intracellular regions may bind directly to each other. To test whether the extracellular regions do so, soluble fusion proteins that contain the ectodomains of CDO and BOC were coupled to either the Fc region of human IgG or to alkaline phosphatase (CDO-Fc, BOC-Fc, CDO-AP, and BOC-AP, respectively). CDO-Fc and BOC-Fc were coupled individually to protein A-Sepharose, and the ability of the AP derivative of the opposite partner to bind was assessed relative to AP itself. As shown in Figure 6A, BOC-AP bound to CDO-Fc, and CDO-AP bound to BOC-Fc, 30- to 40-fold more efficiently than the AP control. Therefore, the CDO and BOC ectodomains can associate in vitro in the absence of other membrane-bound, cellular constituents. To test the intracellular regions, bacterially produced fusion proteins between the intracellular domain of CDO and GST (GST–CDO) and the intracellular domain of BOC and maltose-binding protein (MBP–BOC) were examined in an in vitro binding assay. MBP–BOC bound to GST–CDO, but not to GST (Figure 6B, compare lanes 2 and 4). This binding was efficient, as essentially no MBP–BOC remained in the unbound fraction (Figure 6B, compare lanes 6 and 8).
Fig. 7. A soluble, secreted form of the BOC ectodomain promotes differentiation of C2C12 cells and is inhibited by a dominant-negative form of CDO. (A) Western blot analysis of expression of CDO-Fc and BOC-Fc in C2C12 cell transfectants, (+), transfection with control expression vector; (+), transfection with CDO-Fc and BOC-Fc expression vectors, as indicated. Filters were probed with antibodies to the Fc region of human IgG. (B and E) Western blot analysis of muscle-specific proteins expressed by the indicated C2C12 cell transfectants cultured for the indicated times under differentiation-inducing conditions (see Materials and methods for details). Expression of CDK2 was used as a control. (C) Photomicrographs of C2C12 cell transfectants that were cultured for 3 days under differentiation-inducing conditions, fixed and stained with an antibody to MHC. (D) 293T cells were transiently transfected with expression vectors for CDO and either BOC-Fc or CD164-Fc as shown. Lysates from such cultures were precipitated with protein A-Sepharose as indicated and then analyzed by western blotting with antibodies to CDO or the Fc region of human IgG, also as indicated. Supernatants from the precipitates (Sup) and conditioned medium (CM) from the transfectants were also analyzed by western blotting with the indicated antibodies. Note that the gel shown in the central panel exhibits a ‘smile’ effect.

Taking the data in Figures 5 and 6 together, it is concluded that CDO and BOC form complexes in a cis fashion via association between both their respective extracellular and intracellular domains and that this most likely occurs through direct interactions.

**A soluble BOC ectodomain fusion protein promotes myogenic differentiation and is blocked by a dominant-negative form of CDO**

Stable expression in C2C12 cells of CDO-Fc significantly delays differentiation of these cells, presumably by competition with endogenous CDO for extracellular binding partners, and thus functions as a dominant-negative form of this protein (Kang et al., 1998). It was therefore of interest to assess the effects of BOC-Fc on myogenic differentiation. C2C12 cell derivatives that stably expressed BOC-Fc were generated by transfection (Figure 7A) and tested for their ability to differentiate relative to control transfectants. In striking contrast to previous observations on cells that expressed CDO-Fc, C2C12 cells that expressed BOC-Fc displayed accelerated induction of myogenin, MHC and TnT (Figure 7B). Furthermore, BOC-Fc-expressing cells formed larger myotubes than did the control transfectants [Figure 7C; 82% versus 60% of nuclei in myotubes for BOC-Fc-expressing versus control cells (average values from four experiments)]. Comparable results were obtained when conditioned medium from 293T cells that had been transiently transfected with BOC-Fc was added to naive C2C12 cells, but not when conditioned medium from mock-transfected cells was used (data not shown). These data indicate that, in contrast to what is observed with CDO, the intracellular region of BOC is not essential for its ability to positively regulate differentiation.

If CDO–BOC complexes function as receptors, a potential explanation for the positive effects of BOC-Fc on differentiation is that BOC’s role may be more in recognition of a ligand than in signaling intracellularly, and that BOC-Fc might bind in cis to full-length CDO to form a productive receptor complex. If this is correct, stable association between BOC-Fc and CDO should be detectable. To test this possibility, CDO and BOC-Fc were
transiently expressed in 293T cells, and the cultures were washed several times prior to lysis. Cell lysates were then precipitated with protein A–Sepharose to bring down BOC-Fc and immunoblotted with antibodies to CDO. As shown in Figure 7D (left panel), full-length CDO co-precipitated efficiently with BOC-Fc. Both mature, fully glycosylated CDO and, at lower abundance, incompletely glycosylated forms of CDO were present in the precipitates. The relative molecular weight of the precipitated BOC-Fc was similar to that of BOC-Fc found in the conditioned medium of the transfected cultures (Figure 7D, middle panel). Taken together, these data indicate that the interaction between CDO and BOC-Fc occurred mainly between the mature forms of these proteins and is therefore likely to occur in substantial measure on the cell surface. This conclusion is consistent with the observation, mentioned above, that BOC-Fc supplied via conditioned medium was effective at stimulating differentiation. As a control, CDO was co-expressed transiently with CD164-Fc, an Fc fusion protein harboring the extracellular region of the pro-myogenic cell surface sialomucin, CD164 (Lee et al., 2001). No association between CD164-Fc and CDO was detected, as all the CDO remained in the supernatant of the protein A–Sepharose precipitation (Figure 7D, right panel). It is concluded that, despite lacking transmembrane and intracellular domains, BOC-Fc is able to mimic full-length BOC in its ability to complex with CDO and to enhance myogenic differentiation.

A further prediction of a model in which CDO and BOC form a functional receptor complex, with CDO but not BOC playing an obligatory role intracellularly, is that the negative effects of CDO-Fc on differentiation should predominate over the positive effects of BOC-Fc. To test this prediction, C2C12 cells were transfected with expression vectors for both CDO-Fc and BOC-Fc (Figure 7A) and assessed for their ability to differentiate relative to a vector control and to cells expressing CDO-Fc alone. As previously reported, expression of CDO-Fc delayed and reduced the production of MHC and TnT, and resulted in formation of fewer and smaller myotubes (Figure 7C and E; 28% versus 60% of nuclei in myotubes for CDO-Fc-expressing versus control cells). Combined expression of CDO-Fc and BOC-Fc was at least as effective at inhibiting differentiation as CDO-Fc alone, as assessed both by expression of muscle structural proteins and by myotube formation (Figure 7C and E; 13% of nuclei in myotubes for CDO-Fc- plus BOC-Fc-expressing cells). Thus, the effects of CDO-Fc are predominant over the effects of BOC-Fc. Taken together, the results strongly indicate that BOC is dependent on CDO for activity and, therefore, that CDO–BOC complexes function to regulate myogenic differentiation.

**Discussion**

Determination and differentiation of cells in the vertebrate skeletal muscle lineage require specific cell–cell interactions between muscle precursors, but the cell surface proteins involved are largely unknown. We previously have reported that CDO, an Ig/FNIII repeat family member expressed in muscle precursors in vitro, enhances myogenesis in vivo in a fashion consistent with its playing a role in this and/or related phenomena (Kang et al., 1998). Here we describe the identification of BOC, another receptor-like protein of the Ig/FNIII repeat family, which works together with CDO as a component of a complex that is likely to play a role in the cell–cell interactions that are important in myogenesis.

**CDO and BOC are co-expressed during development, have similar activities and form functional complexes**

Several lines of evidence indicate that BOC plays an important role as a positive regulator of myogenic differentiation and that it does so by a mechanism similar to and involving CDO. This conclusion is based on the observations that: (i) cdo and boc are co-expressed in muscle precursors in the developing mouse embryo and in C2C12 myoblasts; (ii) overexpression of either CDO or BOC in myoblast cell lines enhances differentiation; (iii) oncogenic Ras inhibits differentiation of myoblasts in a manner dependent on down-regulation of both CDO and BOC; (iv) CDO and BOC form complexes in a cis fashion, with two regions of these proteins independently interacting with each other (i.e. the respective extracellular and intracellular domains); and (v) a dominant-negative deletion mutant of CDO blocks the effects of an active form of BOC.

One significant difference between CDO and BOC is that a soluble, secreted form of the extracellular region of CDO (CDO-Fc) functions as a dominant-negative factor to inhibit myogenic differentiation, while an analogous form of BOC (BOC-Fc) promotes this process, similarly to full-length BOC. Thus, the intracellular region of CDO is required for its pro-myogenic effects, while that of BOC is dispensable. Importantly, the negative effects of CDO-Fc on differentiation are predominant over the positive effects of BOC-Fc, strongly arguing that BOC is dependent on CDO for activity in this system and that CDO–BOC complexes are functional. A plausible explanation for these observations is that CDO and BOC function together as components of a receptor, with BOC’s role primarily occurring extracellularly (e.g. binding in trans to a ligand or in cis to additional components of the complex), while CDO’s role includes an obligatory intracellular function (e.g. signaling). This model predicts that BOC-Fc itself would be able to form complexes with CDO and, indeed, this was observed in co-precipitation experiments.

Taking all these data together, an attractive hypothesis is that CDO and BOC form a receptor complex that, in response to engagement with an as yet unidentified ligand, serves to positively regulate the myogenic program. We have speculated previously that CDO might signal to activate MyoD post-translationally (Kang et al., 1998). The observation that BOC, like CDO, exerts its effects in myoblast cell lines without alteration of MyoD levels is consistent with this possibility. We recently have addressed this point experimentally through the use of transient transfection assays with a muscle-specific reporter construct in 10T1/2 fibroblasts (which express boc but not cdo): in these assays, a given level of MyoD is significantly more effective in the presence of co-transfected CDO (F.Cole and R.S.Krauss, unpublished results). In apparent contrast to these results, re-expression of CDO or BOC in C2C12/Ras cells led to induction
of myoD expression. It is worth noting, however, that C2C12/Ras cells retain low levels of MyoD and Myf-5 (Kang et al., 1998), which presumably are kept inactive by post-translational mechanisms (Lassar et al., 1989). C2C12/Ras cells also retain low levels of CDO (Kang et al., 1998). We suggest that ectopic expression of CDO or BOC overrides or reverses Ras-mediated inhibition of the remaining low levels of MyoD and Myf-5 and, consequently, reactivates the nuclear positive feedback network that regulates expression of myogenic bHLH factors and MEF2. After a critical threshold concentration of these proteins is achieved, the ability to differentiate could be restored even in the presence of activated Ras. In this way, CDO and BOC could function principally to activate MyoD post-transcriptionally, yet could indirectly affect MyoD gene expression in C2C12/Ras cells.

Identification of the ligand for a CDO–BOC receptor complex is of very great interest, as this factor would be predicted to be a strong inducer of myogenic differentiation. Furthermore, since CDO and BOC are also co-expressed in neural precursors and other cell types during development (Mulieri et al., 2000, 2002), this factor might have differentiation-inducing activity in a broad range of cell types. CDO-Fc and BOC-Fc bind to the surface of C2C12 cells, suggesting that the ligand may be an integral membrane protein or an extracellular matrix-bound factor (unpublished results). We have not observed binding between CDO and BOC in trans, but cannot rigorously rule out that CDO–BOC complexes recognize each other on apposing cells. However, the ligand or ligands for CDO and BOC may well be heterophilic molecules. This is the case with the DCC and Robo proteins, which represent the subfamilies most closely related to the CDO/BOC subfamily, and which serve as receptors for netrin and slit proteins, respectively (Keino-Masu et al., 1996; Brose et al., 1999). In either case, the developmental expression patterns and activities of CDO and BOC, as well as setting a precedent for members of the Ig superfamily, all suggest that the CDO–BOC complex may function to mediate some of the effects of cell aggregation and high cell density that promote myogenic differentiation.

Models to explain CDO and BOC activities must also ultimately explain the observation that boc mRNA is easily detected in numerous adult tissues, including skeletal muscle, while cdo mRNA is barely detectable in these tissues (Kang et al., 1997). It is unknown whether BOC might function in the absence of CDO under some circumstances, or whether cdo expression might be induced at times of tissue renewal and cell differentiation that have not yet been studied (e.g. during muscle regeneration). Further work is required to address these possibilities.

A new Ig/FNIII subfamily

The five Ig + three FNIII repeat topography of the CDO ectodomain has led to its affiliation with the similarly structured Robo receptors, evolutionarily conserved axon guidance proteins. However, Robo proteins identified in C. elegans, Drosophila and vertebrates are all more closely related to each other than to CDO (Simpson et al., 2000); furthermore, cdo is not expressed in neurons at times of axon outgrowth (Mulieri et al., 2000). These observations raised the question of what the relationship is between CDO and the Robo subfamily. Phylogenetic analysis of genes from several species indicates that, along with two genes identified in the Drosophila genome sequence (CG13756 and CG9211), cdo and boc form a newly identified subfamily, distinct from the robo subfamily. Interestingly, BOC has a 4 + 3 structure and the two Drosophila genes have a 4 + 2 structure, suggesting that the 5 + 3 configuration of CDO may have arisen by convergent evolution with the Robo subfamily, which established this pattern of repeats much earlier. Alternatively, it is possible that a common ancestor had a 5 + 3 structure and boc and the fruit fly 4 + 2 genes lost repeats during evolution. It is worth noting that CDO’s fifth Ig domain, which is absent in BOC, can be alternatively spliced, producing a 4 + 3 CDO protein (Kang et al., 1997). Another difference between the CDO and Robo subfamilies is that Robos are most alike in their first two Ig domains (Simpson et al., 2000), while CDO, BOC, CG13756 and CG9211 are most closely related in their membrane-proximal FNIII repeats. The expression patterns of CG13756 and CG9211 during fly development are not known. However, because binding of ligands for Ig/FNIII receptors can be observed across species barriers (Keino-Masu et al., 1996; Brose et al., 1999), we recently have expressed CG13756 and CG9211 in C2C12 cells. In preliminary experiments, ectopic expression of either gene enhanced myogenic differentiation (unpublished observations), suggesting that an evolutionarily conserved pathway may exist.

Positive feedback loops in myogenesis and their regulation by oncogenic Ras

Myogenesis is a useful model to understand the inverse relationship between cell differentiation and cell transformation. In particular, oncogenic forms of Ras can block differentiation by transcriptional down-regulation of myogenic bHLH factors and by post-translational inhibition of their activity (Lassar et al., 1989). We have shown previously that Ras also inhibits expression of cdo in C2C12 cells, and that this action of Ras is required for its ability to block differentiation of these cells (Kang et al., 1998). Forced re-expression of exogenous cdo in C2C12/Ras cells leads to induction of endogenous MyoD and vice versa, indicating that a positive feedback loop exists between CDO at the cell surface and myogenic bHLH factors in the nucleus (Kang et al., 1998). cdo behaves identically to cdo in this system. Oncogenic Ras down-regulates boc expression, which is restored by exogenous MyoD. Furthermore, forced re-expression of boc induces MyoD and reactivates the differentiation program. Thus, BOC is the second identified cell surface component of an extended positive feedback network that regulates, and is in turn regulated by, the core nuclear network of myogenic transcription factors. Ras therefore blocks differentiation by decreasing expression of multiple components of this feedback system. The fact that forced re-expression of single components of the system (i.e. MyoD, CDO or BOC) can reactivate the entire differentiation program strongly supports the notion of a close mechanistic connection between these proteins, and suggests that there is a balance between Ras-mediated signals that dampen the extended network and the signaling pathways that presumably connect the CDO–BOC complex at the
cell surface with the core nuclear network of myogenic transcription factors. Uncovering the circuitry that underlies the extended feedback loop described here should provide important information about cell differentiation decisions that occur during development and how such decisions go awry in oncogenesis.

Materials and methods

Isolation of boc, cdo and Drosophila cDNAs

A human boc cDNA was isolated by low-stringency screening of a human fetal brain cDNA library (Clontech) with a rat cdo cDNA. Three mouse boc cDNA clones (AW230653, BF385469 and BB586087) were identified subsequently in the EST database and were obtained from ATCC. Xenopus cdo and boc cDNAs were isolated by low-stringency screening of a tadpole head library (provided by A.Hemmata-Brivanlou, Rockefeller University). Two related fruit fly genes were identified by searching the Drosophila genome database with the full-length cDO amino acid sequence. The ORF of one gene (CG9211) was fully annotated, but that of the other was split in two, due to a nested gene (the two partial ORFs were designated CG13756 and CG7894 but the gene is referred to here as CG13756). Two corresponding EST clones (clone ID Nos SD07678 for CG13756 and GH06022 for CG9211) were obtained from Research Genetics. Sequence analysis confirmed that these EST clones contained the entire ORFs of each gene. DNA sequencing was performed at the Mt Sinai DNA Sequencing Core Facility. Searches of the cDNA databases were performed with the BLAST server service, and sequence analysis and alignments were carried out with MacVector (International Biotechnologies, Inc.). The phylogenetic tree was constructed with ClustalW to determine the multiple alignment and then with the PHYLIP program and kirsch methods. DDBJ/EMBL/GenBank accession Nos are: human boc, YA726768; mouse boc, AF388037; Xenopus boc, AF388036; Xenopus cdo, AF388035.

In situ hybridization

A mouse boc cDNA in PCMVSport6 (Life Technologies) was used to make 5'- or digoxigenin-labeled antisense riboprobes for thin section and whole-mount in situ hybridization, respectively. Plasmaid was linearized and antisense riboprobes comprising part of the second and all of the third FNIII repeat, the transmembrane and intracellular domains, and ~600 bp of 3'-untranslated region were synthesized with T7 RNA polymerase. Sense strand riboprobes used as a control did not generate any signal. Thin section and whole-mount in situ hybridizations were performed as described (Sassoon and Rosenthal, 1995).

Cell culture

C2C12 and F3 myoblasts and all cell lines derived from them were cultured as described in Kang et al. (1998). Briefly, cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 15% fetal bovine serum (FBS). For induction of differentiation, cells were switched to DMEM plus 2% horse serum (HME). COS and 293T cells were cultured in DMEM plus either 5 or 10% fetal bovine serum, respectively.

Ectopic expression of BOC, CDO and CDO deletion mutants

A complete human bOC ORF was reconstructed from two overlapping cDNA clones and ligated into either the retroviral expression vector, pBaBePuro (Morgansand and Land, 1990), or the CMV promoter-driven expression vector, pC3DNA3 (Invitrogen). To tag the protein with the flag epitope, the native stop codon was removed and replaced with an oligonucleotide that translates as DPDYKDDDDDKstop. The deletion mutants CDO(MCR1) and CDO(TM1) were derived from a rat cdo cDNA (Kang et al., 1997). CDO(MCR1) has a stop codon inserted after G1001, deleting all but 15 amino acids of the intracellular region. CDO(TM1) contains the first 39 amino acids of the CDO precursor (signal sequence plus 15 amino acids in the region N-terminal to Ig repeat 1) linked directly to the complete transmembrane and intracellular domains. Details of the construction of these mutants are available on request. These cDNAs were ligated into pBaBePuro. Production of recombinant retroviruses and infection of myoblast cell lines were performed as described in Kang et al. (1997). Transient transfections were performed with the FuGene reagent (Roche) according to the manufacturer's instructions.

Soluble BOC fusion proteins

A PCR-derived fragment containing the signal sequence and entire extracellular region of human BOC was subcloned into the Apta-2 or Igta vectors (Bergemann et al., 1995) to produce BOC-AP and BOC-Fc, respectively. Production of these and the analogous CDO-based fusion proteins (Kang et al., 1998) in 293 T cells, and binding assays between them, were performed as described by Bergemann et al. (1995). C2C12 cell derivatives that stably express BOC-Fc and/or CDO-Fc were obtained by co-transfection with pBaBePuro as described (Kang et al., 1998). To assess the differentiation capabilities of C2C12 cells that produced these proteins, it was desirable to avoid transferring the cultures into DM because that would remove the secreted fusion proteins at the same time as the cells received the differentiation signal. As previously described for analysis of CDO-Fc (Kang et al., 1998), cells were plated in GM and held at confluence to allow the medium to become depleted of growth factors.

RNA and protein analysis

Total cellular RNA was isolated with the TRIzol reagent (Life Technologies); northern blot analyses were performed as described by Kang et al. (1997). Expression of boc in adult human tissues was assessed with a Multiple Tissue Northern Blot (Clontech), which was hybridized with human DNA probes as described by Kang et al. (1997).

Western blot analyses were performed as described in Kang et al. (1997). Antibodies used were: anti-MHC (ME20; Developmental Studies Hybridoma Bank); anti-MyoD (Santa Cruz); anti-myogenin (FSD; Santa Cruz); anti-Flag (Sigma); anti-CDK2 (Santa Cruz); anti-Stat (Sigma); anti-CDO (rabbit antiserum against the CDO intracellular region, Zymed); and anti-NCAM (gift of G.Philips, Mt Sinai). A mouse monoclonal antibody (2B3) against the CDO extracellular region was developed with purified CDO-Fc fusion protein as an immunogen. Hybridomas were developed at the Mt Sinai Hybridoma Core Facility by standard procedures; 2B3 was used to detect CDO(ΔCRI) in co-immunoprecipitation experiments. Immunostaining for MHC was performed as described as described by Kang et al. (1998).

To study CDO–BOC complex formation, stably infected C2C12 and transiently transfected 293 T cells were lysed in 50 mM HEPES–HCl pH 7.5/100 mM NaCl/1% Triton X-100 containing 50 mM NaF, 1 mM sodium orthovanadate and protease inhibitors cocktail (Roche) and subjected to co-immunoprecipitation techniques using either rabbit anti-CDO, mouse monoclonal anti-flag or mouse monoclonal anti-CDO (2B3) antibodies. Immunocomplexes were then precipitated with either protein A-Sepharose or goat anti-mouse IgG-conjugated protein G-Sepharose, followed by immunoblot analysis with various antibodies.

To test the ability of the CDO and BOC intracellular regions to bind to each other, PCR fragments encoding these regions of CDO and BOC were subcloned into the pGEX-5X-1 (Amersham Pharmacia) and pMAL-c2 vectors (New England Biolabs) to yield GST–CDO and MBP–BOC, respectively. GST and GST–CDO were purified with glutathione Sepharose 4B (Amersham Pharmacia), and MBP and MBP–BOC were purified with amylose resin. A 20 µl aliquot of GST- or GST–CDO–coupled beads was incubated with 1 µg of MBP or MBP–BOC in 100 mM NaCl/50 mM Tris pH 7.5/1 mM Na2SO4 and 1% Triton X-100. The beads were washed extensively with this buffer, and bound and unbound material analyzed by western blotting and staining of the filter with Ponceau S.

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References


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