AND-34/BCAR3, a GDP Exchange Factor Whose Overexpression Confers Antiestrogen Resistance, Activates Rac, PAK1, and the Cyclin D1 Promoter


ABSTRACT

AND-34 is a murine protein that binds by a cdc25-like GDP exchange factor domain to the focal adhesion docking protein p130Cas. Overexpression of either of the human homologues of AND-34 and p130Cas, BCAR3 and BCAR1, respectively, has been reported to induce resistance to antiestrogens in breast cancer cell lines. Here we show that overexpression of AND-34 leads to activation of the Rho family GTPases Cdc42 and Rac. Consistent with these findings, BCAR3 overexpression induced alterations in F-actin distribution and augmented both autophosphorylation and kinase activity of the Cdc42/Rac-responsive serine/threonine kinase PAK1. p130Cas-associated BCAR3 protein was detected in the estrogen-independent breast cancer cell line Sf-76T, but not in estrogen-dependent MCF7 or ZR-75-1 cells. Stable ZR-75-1 transfectants overexpressing BCAR3, but not vector-only transfectants, grew in the presence of either 4-hydroxy-tamoxifen or ICI 182,780 (4). AND-34 is the murine homologue of BCAR3, sharing 86% identity and 93% homology to BCAR3 at the amino acid level. Remarkably, further analysis of the 80 antiestrogen-resistant clones demonstrated that four of the clones had independent retroviral insertion into the promoter of BCAR1, the human homologue of p130Cas (5). Although the GDP exchange activity of BCAR3 has not been examined, nor has its association with human p130Cas, these seminal studies by Dorssers and colleagues suggest that BCAR3 and BCAR1 constitute part of a signaling cascade that bypasses estrogen dependence in human breast cancer cell lines. In this study, we present evidence that AND-34/BCAR3 confers antigenestrogen resistance through a Rac-mediated pathway.

MATERIALS AND METHODS

Muristerone-Inducible Cell Line. Ecr-293 cells (Invitrogen) were transfected with a pIND-HA-AND-34 construct, and stable clones were selected with 400 μg/ml G418. Inducibility was confirmed in the presence and absence of 1 μM muristerone.

Antibodies. Rabbit sera were immunized with a 19-amino acid BCAR3 peptide, YEK(3)LPKF5SSKLLH(2)REST (QCBI, Hopkinton, MA). The specificity of the resulting polyclonal antisera (anti-BCAR3) was verified by immunoblotting whole cell lysates of Cos7 cells transfected with vector or HA-tagged BCAR3 with anti-HA or anti-YEK.

F-Actin Staining. Retrovirally transduced NIH 3T3 cells were fixed in 3.7% formaldehyde in PBS for 10 min, washed twice with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. The cells were incubated with 5 units of Alexa594-phalloidin (Molecular Probes) for 20 min at room temperature, washed twice with PBS, examined with a Nikon Diaphot fluorescent microscope, and photographed with a Hamamatsu digital camera.

AND-34/BCAR3 and BCAR1, respectively, has been reported to induce resistance to antiestrogens in breast cancer cell lines. Among the resulting 80 tamoxifen-resistant clones, 6 resulted from independent retroviral insertion into the promoter for a novel gene BCAR3. The retroviral insertion was shown to result in the up-regulation of BCAR3 expression and growth of such ZR-75-1 cells in the presence of either 4-hydroxy-tamoxifen or ICI 182,780 (4). AND-34 is the murine homologue of BCAR3, sharing 86% identity and 93% homology to BCAR3 at the amino acid level. Remarkably, further analysis of the 80 antiestrogen-resistant clones demonstrated that four of the clones had independent retroviral insertion into the promoter of BCAR1, the human homologue of p130Cas (5). Although the GDP exchange activity of BCAR3 has not been examined, nor has its association with human p130Cas, these seminal studies by Dorssers and colleagues suggest that BCAR3 and BCAR1 constitute part of a signaling cascade that bypasses estrogen dependence in human breast cancer cell lines. In this study, we present evidence that AND-34/BCAR3 confers antiestrogen resistance through a Rac-mediated pathway.
Rap1A V12 and Rac1 V12 constructs were generously provided by Dr. Lawrence Quilliam (Indiana University School of Medicine).

**RESULTS**

**AND-34/BCAR3 Overexpression Induces Alterations in Cell Morphology and Activates Rac1, Cdc42, and PAK1.** To study the functional effects of AND-34 in cells that do not constitutively overexpress this protein, we obtained stable transfectants of the Ecr-293 mesenchymal kidney cell line in which muristerone induced overexpression of HA-tagged AND-34. After treatment of one such clone, HA-A34/#9, with muristerone, HA-tagged AND-34 could be detected easily in whole cell lysates with either an anti-HA monoclonal antibody or with polyclonal anti-AND-34 antiserum (Fig. 1A). Low levels of a protein immunoreactive with anti-HA and polyclonal anti-AND-34/BCAR3 were detected in the noninduced Ecr-293 cells (Fig. 1A). After the addition of muristerone, 10–20% of HA-A34/#9 cells developed long cellular processes (Fig. 1B). Time-lapse cinematography revealed that such processes developed as the cell body moved, leaving a retraction fiber behind (data not shown). The number of HA-A34/#9 cells with cell extensions greater than 1 cell diameter in length increased from 2.9 to 11.0 cells/high-powered field (P < 0.001) after muristerone treatment. Because control immunofluorescence studies of permeabilized cells verified that essentially no uninduced cells and all muristerone-treated cells expressed HA-BCAR3 (data not shown), the presence of long cell extensions in BCAR3-overexpressing HEK-293 cells may vary as a function of cell-cell contact or motility.

To examine the effects of AND-34 expression in a model system in which cytoskeletal regulation has been well studied, we transduced NIH 3T3 cells with a MSCV retrovirus that drives expression of both GFP and HA-tagged AND-34 (AND-34-RV) or the same retrovirus lacking HA-AND-34 (CT-RV). When F-actin in the GFP-positive transduced cells was examined by fluorescence microscopy using Alexa594-phallolidin, we noted clear stress fibers and modest numbers of lamellipodia in the CT-RV-transduced cells (Fig. 2, A and B). In contrast, AND-34-RV-transduced NIH 3T3 cells contained both a reduced number and a less organized pattern of parallel stress fibers (Fig. 2, C and D). AND-34-RV-transduced cells also showed more pronounced membrane “ruffles” or lamellipodia than CT-RV-transduced cells. As a control, filopodia (Fig. 2E) and lamellipodia (Fig. 2F) were examined in NIH 3T3 cells transiently cotransfected with plasmids driving the expression of GFP and either constitutively activated CDC42 (Cdc42 L61) or Rac (Rac V12), respectively. As shown in Fig. 2, AND-34-RV-transduced NIH 3T3 cells more closely resembled cells containing activated Rac. No significant morphological alterations were observed in NIH 3T3 cells transiently transfected with RalA V23, a constitutively active form of Ral (data not shown).

Because similar morphological changes have been observed after overexpression of activated Rho family GTPases, we performed pull-down assays to examine such activation in HA-A34/#9 cells induced

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**Fig. 1. Induction of AND-34 overexpression in HEK-293 cells is associated with alterations in cell morphology. A, an Ecr-293 cell line stably transfected with HA-tagged AND-34 (HA-A34/#9) was incubated for 24 h in the presence (+) or absence (−) of 1 μM muristerone, followed by lysis and immunoblot analysis with monoclonal anti-HA antibody (left panel) or polyclonal anti-AND-34 antibody (right panel). B, HA-A34/#9 cells were grown in media with (Induced) or without (Control) muristerone as per A, followed by microscopy. In cultures of similar cell density, the tissue culture plate was divided into eight equal regions, and in each region, the number of cells with cell extensions of >1 cell diameter in length (right panels) was quantitated. The observed increase in cell extensions (column graph at bottom right) was significant at 0.01% (**) by Student’s t test.**
to overexpress AND-34 (9, 10). We used a GST-PAK-RBD construct to selectively isolate the GTP-bound forms of transiently transfected FLAG epitope-tagged Cdc42 and Rac1. A GST-Rhotekin-RBD construct was used to isolate endogenous GTP-bound HA-tagged RhoA (7). Significant levels of GTP-bound Cdc42 (Fig. 3A) and Rac (Fig. 3B) but not RhoA (Fig. 3C) were present in induced HA-A34/#9 cells, but not in noninduced HA-A34/#9 cells. In five of six experiments, transient transfection of BCAR3, the human homologue of AND-34 (86% identity), induced activation of endogenous Rac1 in 293T cells (Fig. 3D). The Rac1 activation observed in these experiments was less than that in the inducible cell line system, most likely due to a lower percentage of cells expressing BCAR3 after transient transfection as well as relatively high levels of activated endogenous Rac1.

Given our prior findings that AND-34 augmented the ratio of GTP:GDP-bound Ral and Rap1 in Cos7 cells, we sought to determine whether these GTPhases might be responsible for the Rac activation we observed in muristerone-treated HA-A34/#9 cells. Transfection of 293T cells with constitutively active forms of these GTPhases, RalA V23 and Rap1A V12, failed to induce Rac activation as judged by pull-down assays (Fig. 3E). As a control, Ral and Rap1 pull-down assays confirmed augmented levels of GTP-bound RalA and Rap1A in 293T cells transfected with these constitutively active constructs (Fig. 3E). These studies suggest that AND-34- and BCAR3-mediated Rac activation occurs independently of Ral or Rap1.

GTP-bound Rac and Cdc42 bind to a CRIB domain in the p21-activated serine threonine kinase PAK1 (11), inducing PAK1 autophosphorylation at serines 199 and 204 and augmenting kinase activity. We therefore examined PAK1 in 293T cells transiently transfected with wild-type BCAR3 and a GST-PAK1 expression construct. After transfection, GST-PAK1 was isolated with glutathione-Sepharose beads and examined for both phosphorylation status and kinase activity. As judged by kinase assay using myelin basic protein as a substrate, overexpression of BCAR3 led in a dose-dependent fashion to GST-PAK kinase activation, as did cotransfection with constitutively active Rac V12 (Fig. 4A). Similarly, using a phosphospecific monoclonal antibody, overexpression of BCAR3 augmented levels of GST-PAK1 autophosphorylated at serines 199 and/or 204 (Fig. 4A). BCAR3 or Rac V12 transfection particularly augmented levels of a more slowly migrating GST-PAK1 species, consistent with phosphorylation of additional residues. Western analysis of whole cell lysates demonstrated comparable expression levels of GST-PAK1 auto- and/or Rac V12 transfection in the presence or absence of cotransfected BCAR3. In contrast, transfection with constitutively active Rac V12 diminished levels of GST-PAK1 (Fig. 4A).

BCAR3 Associates with Human p130Cas in an Estrogen-Independent Breast Cancer Cell Line. To examine the potential role of Rac or Cdc42 activation in BCAR3-mediated antiestrogen resistance, we examined two human breast cancer cell lines, 578-T and ZR-75-1.
in murine cells, at least a fraction of BCAR3 is constitutively associated with p130Cas (BCAR1) in human 578-T cells.

To study the effect of BCAR3 on antiestrogen sensitivity in ZR-75-1 cells, we generated a plasmid construct in which an NH2-terminal HA epitope-tagged full-length BCAR3 cDNA was driven by a constitutive cytomegalovirus promoter. Stable transfectants of ZR-75-1 cells were obtained with both HA-tagged BCAR3 (BCAR3 #2, #4, and #6) and with the vector alone (CT #1, #3, and #4). The three BCAR3-overexpressing ZR-75-1 stable transfectants contained significantly greater numbers of long, branched cellular processes than the three vector-only transfectants (P < 0.0001 by one-factor ANOVA). As expected, none of the three vector-only stable transfectants grew in 100 nM ICI 182,780. In contrast, all three of the BCAR3-overexpressing clones grew well in 100 nM ICI 182,780 (Fig. 4C). The cell doubling time of the three BCAR3-overexpressing transfectants was not significantly different from that of the three vector-only transfectants when grown in normal media (data not shown). After the addition of antiestrogen to the culture media, growth of all of the clones gradually diminished over the first week. Subsequently, however, the growth rate of the BCAR3-overexpressing clones increased such that they were growing at close to (BCAR3 #2 and #6) or moderately slower than (BCAR3 #4) the doubling time of cells grown in the absence of the antiestrogen (data not shown). To establish whether the ability of BCAR3 to activate Rac1 might underlie its ability to confer antiestrogen resistance to ZR-75-1 cells, we isolated stable transfectants of this cell line containing either a constitutively active form of Rac1, FLAG-tagged Rac1 V12, or vector alone. Western analysis confirmed overexpression of Rac V12. Whereas the three control vector lines failed to grow in 100 nM ICI 182,780, the two Rac1 V12 cell lines grew robustly (Fig. 4D).

**BCAR3 Overexpression Activates the Cyclin D1 Promoter in a Rac- and PAK1-dependent Manner.** Cyclin D1 is known to play an important role in estrogen-mediated breast cancer cell growth, and antiestrogens block cyclin D1 expression. To analyze the effects of BCAR3 on cyclin D1 expression in transient transfection assays, we used a second estrogen-dependent cell line, MCF7, that allowed greater transfection efficiency than ZR-75-1 cells. By Western analysis, transient transfection of BCAR3 into MCF7 cells augmented cyclin D1 expression. In five experiments, cotransfection of the cyclin D1 construct with BCAR3 markedly augmented luciferase activity relative to cotransfection with empty vector, regardless of the presence or absence of 100 nM ICI 182,780 (Fig. 5B). To determine the minimal promoter length required for BCAR3-mediated cyclin D1 transcriptional activation, we examined two additional cyclin D1 luciferase constructs containing 163 or 66 bp 5’ of the cyclin D1 transcriptional initiation site. Whereas BCAR3 augmented transcription of the 1745- and 163-bp constructs, no significant transcriptional activation of the 66-bp construct was detected (Fig. 5C). Thus, transcription factor binding sites 5’ of bp 66 are likely to play a critical role in BCAR3-mediated cyclin D1 promoter activation.

Because the work described above suggests that overexpression of AND-34 or BCAR3 activates Rac and the serine/threonine kinase PAK1, we sought to determine whether such activation might play a role in BCAR3-mediated cyclin D1 promoter activation. We cotransfected BCAR3 and the 1745-bp cyclin D1 luciferase construct with either control vector, dominant negative Rac1(N17), or dominant negative PAK1(K297A) (12). Both Rac1(N17) (P < 0.008) and PAK1(K297A) (P < 0.001) reduced BCAR3-mediated cyclin D1 promoter activation (Fig. 5, D and E, respectively). In contrast, cotransfection with a dominant negative
form of MEK1(S218A,S222A) had no inhibitory effect on BCAR3-mediated cyclin D1 promoter activation (Fig. 5F; Ref. 13). Given that the dominant negative PAK construct used has an intact CRIB domain that could theoretically sequester activated Cdc42 and Rac from other potential effector proteins, we next cotransfected BCAR3 with PID (residues 83–139) that does not bind Rac or Cdc42 but can inhibit PAK1 activation (8). BCAR3-mediated cyclin D1 promoter activation was markedly inhibited by the wild-type PID construct, but not by a PID construct inactivated by replacing leucine 107 with phenylalanine (Fig. 5G; Ref. 14). These experiments suggest that BCAR3-mediated antiestrogen resistance may be mediated, at least in part, by Rac and PAK1-induced cyclin D1 promoter activation.

**DISCUSSION**

In this study, we demonstrate that overexpression of AND-34 and BCAR3 can activate Rac and/or Cdc42 signaling pathways. Given that AND-34 and BCAR3 have domains homologous to the cdc25 domain of GEFs, it is likely that such Rho family GTPase activation occurs indirectly as a result of initial activation of a Ras subfamily GTPase. However, although there is ample precedent for cross-talk between Ras and Rho subfamily GTPases, to our knowledge this is the first report in which overexpression of a GEF with a cdc25-like domain (but no Dbl domain) results, directly or indirectly, in activation of Rho family GTPases (15, 16). One potential mechanism by which a Ras subfamily GEF might indirectly activate Rho subfamily GTPases is through activation of PI3K. The Rac/Cdc42 GEF activity of several Dbl domain-containing GEFs such as Sos-1, Vav, and PIX is activated by interaction of their pleckstrin homology (PH) domains with phosphatidylinositol-(3,4,5)-triphosphate (PIP3), the product of PI3K (17–20). Alternatively, AND-34 or BCAR3 overexpression could lead to kinase activation and the phosphorylation and activation of a Rho family GEF. Tyrosine phosphorylation of the Dbl family GEF Vav occurs after stimulation of some 35 membrane receptors and has been reported to induce either activation or down-modulation of Vav activity (21, 22). We are currently examining whether AND-34 or BCAR3 overexpression activates PI3K or leads to tyrosine phosphorylation of known Rac/Cdc42 GEFs.

AND-34/BCAR3 could induce Rho subfamily GTPase activation by acting as an adapter protein rather than as a GEF. AND-34 and BCAR3 contain both an SH2 domain and a proline-rich region that could serve to recruit either a Rho subfamily GEF or an enzyme that activates such a GEF. As discussed above, overexpression of p130Cas as well as BCAR3 results in tamoxifen resistance, raising the question of whether p130Cas achieves this effect by regulating the GEF activity of BCAR3 or through another protein. Several reports have linked p130Cas to Rac1 activation (reviewed in Ref. 23). p130Cas overexpression enhances epithelial cell migration through a CrkII- and Rac1-dependent pathway (24). Whereas the SH2 domain of CrkII binds inducibly to tyrosine-phosphorylated p130Cas, the SH3 domain of CrkII binds to several proteins including the Rap1 GEF C3G and DOCK180 (25, 26). DOCK180 binds Rac1, but not RhoA or Cdc42, and DOCK 180 overexpression leads to increased levels of GDP-bound Rac (27). Given this background, it is possible that p130Cas overexpression could induce Rac activation by pathways independent of BCAR3. However, our observation of Cdc42 activation by AND-34 overexpression suggests that AND-34/BCAR3-mediated Rho subfamily GEF activation is unlikely to be occurring by a
DOCK180-mediated pathway. Finally, AND-34/BCAR3 might act directly as a Rac/Cdc42 GEF. Although AND-34/BCAR3 has homology to cdc25, the homology is distant enough (18% identity) to raise the possibility that this enzyme may have dual Ras and Rho family GEF activity. Studies examining the in vitro GEF activity of AND-34 will be required to resolve this question unequivocally.

A number of reports have previously implicated Rac in regulation of the cyclin D1 promoter, supporting our hypothesis that Rac itself is responsible for BCAR3-mediated antiestrogen resistance. Studies using bovine tracheal myocytes, NIH 3T3 cells, HeLa cells, and Cos1 cells have reported that Rac activation can induce cyclin D1 synthesis and progression through G1 by a superoxide- and NF-κB-mediated pathway (28–31). Although these studies have implicated p67phox as a necessary effector for Rac-mediated cyclin D1 promoter activation, they have not shown that activation of this p67phox/superoxide/NF-κB pathway is sufficient for this end point. In fact, the multiple transcription factor binding elements that have been shown to be required for cyclin D1 promoter activation suggest that robust activation of this promoter requires the synchronous activation of several signal transduction pathways. Our examination of BCAR-mediated effects on a series of cyclin D1 promoter deletion constructs suggests that critical transcription factor-binding elements are contained in the region from −66 to −163. Prior studies have documented several functionally important DNA transcription factor-binding sites in this region, including TCF/LEF (−75), E2F (−127), and SP1 (−143 (32–34)). We are currently examining the role of these transcription factors, as well as the p67phox/superoxide/NF-κB pathway, in BCAR3-mediated cyclin D1 promoter activation.

Van der Flier et al. (35) have examined the expression of BCAR1 (p130Cas) in breast cancer specimens and found that overexpression of BCAR1 was associated with short disease-free interval and survival. Examination of BCAR3 in normal and malignant human breast specimens will be necessary to determine whether BCAR3 overexpression induces antiestrogen resistance in patients with breast cancer. Regardless of the outcome of such studies, our work suggests that activation of a Rac and PAK1 signaling pathway may play a role in antiestrogen resistance in human breast cancer.

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