Breast Cancer Anti-estrogen Resistance 3 (BCAR3) Protein Augments Binding of the c-Src SH3 Domain to Crk-associated Substrate (p130cas)*

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Background: BCAR3 binds to p130cas, a known substrate of Src.

Results: BCAR3 augments binding of the Src SH3 domain to p130cas as well as p130cas tyrosine phosphorylation in a BCAR3-p130cas complex-dependent manner.

Conclusion: Formation of a BCAR-p130cas complex enhances Src SH3 domain binding to p130cas and p130cas substrate domain tyrosine phosphorylation.

Significance: These results suggest a molecular basis for BCAR3-mediated enhancement of cell motility.

The focal adhesion adapter protein p130cas regulates adhesion and growth factor-related signaling, in part through Src-mediated tyrosine phosphorylation of p130cas. AND-34/BCAR3, one of three NSP family members, binds the p130cas carboxyl terminus, adjacent to a bipartite p130cas Src-binding domain (SBD) and induces anti-estrogen resistance in breast cancer cell lines as well as phosphorylation of p130cas. Only a subset of the signaling properties of BCAR3, specifically augmented motility, are dependent upon formation of the BCAR3-p130cas complex. Using GST pull-down and immunoprecipitation studies, we show that among NSP family members, only BCAR3 augments the ability of p130cas to bind the Src SH3 domain through an RPLPSPP motif in the p130cas SBD. Although our prior work identified phosphorylation of the serine within the p130cas RPLPSPP motif, mutation of this residue to alanine or glutamic acid did not alter BCAR3-induced Src SH3 domain binding to p130cas. The ability of BCAR3 to augment Src SH3 binding requires formation of a BCAR3-p130cas complex because mutations that reduce association between these two proteins block augmentation of Src SH3 domain binding. Similarly, in MCF-7 cells, BCAR3-induced tyrosine phosphorylation of the p130cas substrate domain, previously shown to be Src-dependent, was reduced by an R743A mutation that blocks BCAR3 association with p130cas. Immunofluorescence studies demonstrate that BCAR3 expression alters the intracellular location of both p130cas and Src and that all three proteins co-localize. Our work suggests that BCAR3 expression may regulate Src signaling in a BCAR3-p130cas complex-dependent fashion by altering the ability of the Src SH3 domain to bind the p130cas SBD.

The focal adhesion protein p130cas (Crk-associated substrate) was first identified as a tyrosine-phosphorylated protein in p47 v-Crk and p60 v-Src transformed cell lines (1). p130cas contains an amino-terminal SH3 domain followed by a “substrate domain” with 15 YXXP motifs, which are the principal site of Src-mediated tyrosine phosphorylation. The p130cas SH3 domain binds to pp125 FAK and related adhesion focal tyrosine kinase, tyrosine kinases whose activity is regulated by integrin signaling (2, 3). Src and other Src family tyrosine kinases are recruited to p130cas in part by Src SH2 domain binding to the autophosphorylation site of FAK, Tyr-397 (4). p130cas substrate domain phosphorylation by Src in turn leads to recruitment of the SH2 domain-containing adapter protein CrkII. Integrin signaling also enhances CrkII association through its SH3 domain with the atypical Rac GDP exchange factor DOCK180, thus assembling a p130cas complex that serves as a molecular switch for cell migration (5–7).

Although p130cas substrate domain tyrosine phosphorylation is clearly of importance to regulation of cell motility, the precise mechanism by which Src is activated to carry out such phosphorylation remains controversial. “Pull-down” studies using glutathione S-transferase (GST)-Src SH2 and GST-Src SH3 domains have demonstrated that a carboxyl-terminal region of p130cas designated the “Src-binding domain” (SBD) contains binding sites for both the Src SH2 domain at Tyr-762 and for the Src SH3 domain at an adjacent RPLPSPP motif (8, 9). Whereas in rat 3Y1 fibroblast cells Src SH2 domain association with p130cas required co-expression of v-Crk or v-Src, association of the Src SH3 domain with p130cas in these cells was constitutive. Mutation of the RPLPSPP motif to RLGSPP resulted in a marked reduction in p130cas-associated kinase activity in v-Crk-transformed cells, suggesting that Src associated with the p130cas SBD may contribute to p130cas substrate domain phosphorylation (8). In contrast to these results, work

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2 The abbreviations used are: SH3, Src homology 3; SH2, Src homology 2; FAK, focal adhesion kinase; Src CA, constitutively active Y528F Src; SBD, Src-binding domain; NSP, novel SH2-containing protein; FAT, focal adhesion-targeting; GEF, guanine nucleotide exchange factor.
BCAR3 Regulates Src SH3 Domain Binding to p130\textsuperscript{cas}

by another group in COS-7 cells reported that mutation of the RPLP0/SPP motif to RAAASPP failed to alter the ability of transfected FAK and n-Src to induce p130\textsuperscript{cas} tyrosine phosphorylation. In these studies, p130\textsuperscript{cas} substrate domain phosphorylation was instead shown to require Src binding to phosphorylated FAK Tyr-397 (10). Of note, n-Src is a neuronal specific splice variant of Src that has a six-amino acid insertion in the SH3 domain that alters Src SH3 domain binding specificity.

Three highly related novel SH2-containing protein (NSP) family members, NSP1, NSP2/AND-34/BCAR3 (BCAR3), and NSP3/SHEP1/CHAT (NSP3), which have an amino-terminal SH2 domain and a carboxyl-terminal domain with modest homology to the Cdc25 homology fold of Ras GDP exchange factors, bind constitutively to the carboxyl-terminal focal adhesion-targeting (FAT) domain of p130\textsuperscript{cas} (11–16). A random retroviral insertional mutagenesis study to identify genes whose altered expression induces anti-oestrogen resistance in estrogen-dependent breast cancer cell lines identified both BCAR3 and p130\textsuperscript{cas} as genes whose augmented expression confers tamoxifen resistance (17, 18). Subsequent studies demonstrated that although all three NSP family members activate Rac and Cdc42 indirectly by a PI3K-dependent mechanism, only BCAR3 induced activation of cyclin D1 promoter luciferase constructs as well as resistance to the pure ER antagonist ICI 182,780 (19–21). As judged by tyrosine phosphorylation of paxillin and cortactin, Riggins et al. (22) reported that co-transfection with BCAR3 and p130\textsuperscript{cas} enhances Src activation in COS-1 cells relative to transfection with p130\textsuperscript{cas} alone. BCAR3 also regulates motility in both fibroblasts and breast cancer epithelial cells (22–24). BCAR3 knock-out mice undergo post-natal ophthalmic lens rupture, suggesting a role for BCAR3 in maintaining the integrity of the lens capsule (25).

Because BCAR3 and p130\textsuperscript{cas} both can regulate cell motility and breast cancer cell line estrogen-independent cell growth, formation of the BCAR3-p130\textsuperscript{cas} complex would be expected to be required for BCAR3-mediated signaling. Surprisingly, in recent studies utilizing an R743A mutant form of BCAR3 that is unable to form a complex with p130\textsuperscript{cas}, we found that many characterized downstream events associated with BCAR3 overexpression are independent of such complex formation (24). Similarly, in the study by Riggins et al. (22) described above, BCAR3-induced Src activation was reported to occur independently of BCAR3 association with p130\textsuperscript{cas}. In contrast, BCAR3-induced augmentation of cellular motility is reduced in fibroblasts expressing the complex formation-defective BCAR3 R743A (24). The molecular mechanisms underlying BCAR3-p130\textsuperscript{cas} complex-dependent and -independent signaling remain unresolved.

In a study examining a BCAR3-induced reduction in p130\textsuperscript{cas} PAGE migration, we determined that BCAR3 expression regulates late phase adhesion-dependent p130\textsuperscript{cas} phosphorylation in an actin filament-dependent manner (26). Three sites of p130\textsuperscript{cas} serine phosphorylation were identified, one of which was located within the previously characterized Src SH3 domain binding site, RPLPSPP. Given this observation, we sought to determine whether expression of BCAR3 could alter the ability of the Src SH3 domain to bind to p130\textsuperscript{cas}. Here, we report that BCAR3 expression augments both Src SH3 domain binding to p130\textsuperscript{cas} and tyrosine phosphorylation of the p130\textsuperscript{cas} substrate domain in a BCAR3-p130\textsuperscript{cas} complex-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The following antibodies were used in this work: rabbit polyclonal anti-BCAR3 (Bethyl) and anti-Src (SRC2; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse monoclonal anti-hemagglutinin (HA) (Covance); anti-p130\textsuperscript{cas} (BD Biosciences); and anti-phospho-Tyr-100, rabbit anti-phospho-p130\textsuperscript{cas} (Tyr-165), and rabbit anti-phospho-p130\textsuperscript{cas} (Tyr-410) (Cell Signaling Technology).

**Mammalian Expression Plasmids and Cell Lines**—Mammalian expression plasmids pUS- Src and pUS-SrcY528F (constitutively active Src) were purchased from Upstate Biotechnology. Vectors expressing wild type and mutant HA-BCAR3 and NSP proteins were described previously (19, 21, 24, 26). Plasmids used for expression of HA-p130\textsuperscript{cas} and HA-p130\textsuperscript{cas} (RLLG) proteins were as described (15). The MCF-7 cell lines stably transduced with HA-NSP-1, HA-BCAR3, and HA-NSP-3 have been described previously (21).

**Mammalian Cell Culture, Transient Transfections, and Cell Lysis**—MCF-7, BCAR3/MCF-7 (II-6 cells), and COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Mediatech, Inc.) supplemented with 10% heat-treated fetal calf serum (Biomeda), 2.2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (27). Transient transfection of cultured cells was performed by mixing plasmid DNA with Lipofectamine 2000 (ratio 1:3 (μg of DNA/μl of Lipofectamine)) in quantities needed to treat duplicate 100-mm culture plates with a subconfluent growth of cells. After 18 h of growth in transfection medium, cells were washed with ice-cold phosphate-buffered saline (PBS). Cell lysates were prepared by scraping cell monolayers on duplicate plates into 750 μl of buffer A (20 mM Hepes, pH 7.4, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 50 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM benzamidine, 1 mM DTT, and 1 mM PMSF) on ice. Cell lysates were clarified using Bradford reagent (Bio-Rad). For immunoprecipitation, pull-down assays, and expression analysis, lysates were normalized to approximately equal concentration of protein.

**Immunoprecipitation, SDS-PAGE, Western Transfer, and Immunoblotting**—For immunoprecipitation, 500 μg of cellular protein was mixed end-over-end with 10–20 μl of protein G-Sepharose (GE Healthcare) and designated antibodies for 3 h at 5 °C. Sepharose beads were collected by centrifuging samples at 20,800 × g for 30 min at 5 °C. Protein concentrations of the clarified cell lysates were measured using Bradford reagent (Bio-Rad). For immunoprecipitation, pull-down assays, and expression analysis, lysates were normalized to approximately equal concentration of protein.
BCAR3 Regulates Src SH3 Domain Binding to p130\textsuperscript{cas}

from Dr. Todd Holmes (University of California, Irvine). A pGEX plasmid encoding the SH3 domain of p130\textsuperscript{cas} was from Dr. Kathrin Kirsch (Boston University School of Medicine). The SH3 domain of mouse p85\textsuperscript{cas} was amplified from expression vector pRSV-Myc-p85\textsuperscript{cas}, a gift from Dr. Zhijun Luo (Boston University School of Medicine), using Pfu DNA polymerase (Stratagene), forward primer (5'-AGTGCAAGGGCTACCGAG-3') and reverse primer (5'-TGAAATCTTTTTCCTTCCAATG-3'). p85\textsuperscript{cas} SH3 forward and reverse primers also had BamHI and NotI restriction enzyme sequences at their 5' termini, respectively. The PCR product was digested with BamHI-NotI (New England Biolabs) and ligated into BamHI-NotI-digested pET-6P-1 (Invitrogen).

Expression and Affinity Purification of Recombinant Proteins—Plasmids encoding GST and GST-tagged recombinant SH3 and SH2 domain proteins were used to transform Escherichia coli strain DH5\textalpha (Invitrogen) as recommended by the manufacturer. Transformed bacteria were cultured in LB containing 100 \mu g/ml ampicillin to midphase of linear growth, upon which time isopropyl-1-thiol-\beta-galactopyranoside was added to the culture to a concentration of 0.5 mM to induce recombinant protein expression. Isopropyl-1-thiol-\beta-galactopyranoside-treated bacterial cultures were grown at room temperature overnight. Bacteria were harvested by centrifugation, and bacterial pellets were lysed using a lytic, non-mechanical method. Bacterial pellets were suspended in lysis buffer B (25 mM Tris-HCl buffer, pH 7.6, containing 100 mM NaCl, 1% Triton X-100, 0.1 mM EDTA, and 1 mM DTT). Egg white lysozyme (1 mg/ml; Sigma-Aldrich) was added, and the mixtures were incubated on ice for 45 min. MgCl\textsubscript{2} (10 mM) and DNase I (25 \mu g/ml) were added and incubated on ice for 20 min. The cytoplasmic fraction of bacterial lysates were prepared by centrifugation at 100,000 × g and mixed with glutathione-Sepharose 4B (GE Healthcare) resin for 3 h. After mixing, Sepharose resins were washed sequentially with buffer C (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 1 mM benzamidine), high salt buffer D (100 mM Tris-HCl, pH 7.6, 1 M NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 1 mM benzamidine), and buffer E (20 mM Heps, 100 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM benzamidine, and 1 mM PMSF). Proteins bound to washed Sepharose resins were eluted with 0.2 M reduced glutathione (Sigma) in buffer E. Eluants were transferred to dialysis tubing (Spectra/Por\textsuperscript{®}; molecular mass cut-off 12–14 kDa), dialyzed overnight against two changes of buffer C containing 50% glycerol, and kept at −20 °C for long term storage. Protein concentrations of the dialysates were determined using Bio-Rad protein assay reagent. Purity of the protein in the dialysates was analyzed by SDS-PAGE and assessed to be >97% by Coomassie Blue staining.

GST Pull-down Assays and SDS-PAGE—Protein lysates prepared from non-transfected, transiently transfected, or transduced cells were normalized to equal concentrations of 1 mg of protein/ml in lysis buffer. GST pull-down samples were assembled by suspending 10–20 \mu l of GSH-Sepharose beads with 1 ml of protein lysate (1 mg) to which 15 \mu g of designated GST-tagged protein was added. Samples were tumbled end-over-end for 2 h at 5 °C. Proteins complexed to GSH-Sepharose beads were recovered by centrifuging samples at 1000 × g for 5 min at 5 °C. Supernatants of the samples were discarded, and Sepharose pellets were washed three times in lysis buffer and three times in lysis buffer containing 250 mM NaCl and suspended in 2× SDS-PAGE sample buffer. Samples were heat-denatured at 100 °C for 5 min and applied to wells of SDS-polyacrylamide gels prepared with 8 and 12% discontinuous resolving acrylamide gels, ½ and ½ length, respectively. After electrophoretic separation, proteins in the 8% acrylamide resolving gels were Western transferred onto PVDF membranes for immunoblot analysis as described above, and proteins retained in the 12% acrylamide resolving gels were stained with Coomassie Blue G-250 (Sigma-Aldrich).

Generation of Src Lentiviral Constructs and Lentiviral Transduction—The insert from the plasmid pUSE-Src wild type and Y528F was subcloned by PCR and inserted into the NotI-BamHI sites of lentiviral vector pHAGE2-FullEFla-ZsGreen-IRES-dTomatoW (23). This construct plus packaging plasmids were transfected into HEK-293T cells in 10-cm plates at 50–70% confluence using Fugene 6 (Roche Applied Science). Medium containing packaged phage particles was harvested, and phage particles were concentrated by centrifugation (SW28 Beckman rotor 1.5 h at 16,500 rpm). For lentiviral transduction, 50 \mu l of the concentrated lentiviral supernatants was added with Polybrene (final concentration 8 \mu g/ml) to MCF-7 and BCAR3/MCF-7 cells cultured in 6-well plates, followed by centrifugation of the plates at 800 × g for 45 min. The transduced cells were trypsinized and resuspended in DMEM with 3% FCS and sorted for dTomato in a MoFlo apparatus (Beckman Coulter). Src overexpression was confirmed by Western analysis.

Immunofluorescent Microscopy—For immunofluorescence studies, cell lines were cultured on fibronectin-coated coverslips (BD Biosciences) as described previously and fixed using 3.7% paraformaldehyde at 37 °C (24). Following incubation with primary antibodies, including anti-c-Src polyclonal antibodies, anti-p130\textsuperscript{cas} mouse monoclonal antibody, and anti-HA mouse monoclonal antibody, cells were probed with Alexa Fluor 350 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse secondary antibodies (Invitrogen). All antibodies were diluted 1:100 in TBS with 2% BSA. After incubation with the antibodies, cells were rinsed several times in TBS prior to being mounted on slides with either Prolong Gold anti-fade reagent with DAPI or Slow Fade Gold without DAPI (Invitrogen) and allowed to cure overnight. Imaging was performed on either a Nikon TE2000-E microscope or a Zeiss LSM 710 Live Duo scan confocal microscope. Images were captured using NIS Elements (Nikon) software and processed using ImageJ (National Institutes of Health) and Photoshop (Adobe).

RESULTS

BCAR3 Expression Augments Src SH3 Domain Association with the RPLP Motif of p130\textsuperscript{cas}—To examine the effect of BCAR3 expression on Src domain interaction with p130\textsuperscript{cas}, purified GST, GST-Src SH2 domain, or GST-Src SH3 domain were incubated with lysates from MCF-7 cells or a stable HA-BCAR3 transfectant of MCF-7, BCAR3/MCF-7 cells. Only GST-Src SH3 associated with endogenous p130\textsuperscript{cas}, and this occurred only in the presence of HA-BCAR3 expression in
BCAR3 Regulates Src SH3 Domain Binding to p130cas

A. GST GSH-Sepharose Pulldown

|          | GST | GST-SH2 | GST-SH3 | MCF-7 | BCAR3/MCF-7 |
|----------|-----|---------|---------|-------|-------------|
|          |     |         |         | +     | +           |
|          | +   | +       | +       | +     | +           |

B. GST GSH-Sepharose Pulldown

|          | GST | GST-SH3 | MCF-7 | BCAR3/MCF-7 |
|----------|-----|---------|-------|-------------|
|          |     |         | +     | +           |
|          | +   | +       | +     | +           |

To confirm BCAR3-induced association of the Src SH3 domain with p130cas using overexpressed epitope-tagged p130cas, a GST pull-down assay was performed on MCF-7 or BCAR3/MCF-7 cells transiently transfected with HA-tagged p130cas. GST-Src SH3 robustly associated with HA-tagged p130cas only in lysates from BCAR3/MCF-7 cells (Fig. 1B). Because BCAR3 induces less protein phosphorylation of transiently transfected HA-p130cas than endogenous p130cas, this experiment also demonstrated that the Src SH3 domain is able to associate with both the slowly and rapidly migrating forms of p130cas in the presence of BCAR3 expression.

The Src SH3 domain has previously been reported to associate with an RPLPSPP motif in the p130cas SBD domain (8). To determine whether BCAR3-mediated enhancement of Src SH3 binding to p130cas occurred at this site, a pull-down assay was performed with a form of HA-p130cas in which this motif was mutated to RLGS. BCAR3 expression in BCAR3/MCF-7 cells failed to enhance association of the Src SH3 domain with the RLGS mutant form of p130cas (Fig. 1B).

BCAR3-induced Binding of the Src SH3 Domain to p130cas is Not Altered by Mutation of p130cas Serine 643—In previous work, we observed BCAR3-mediated serine phosphorylation of human p130cas (26). Mass spectrometry of endogenous p130cas from MCF-7 cells identified phosphorylation of serine 639, which corresponds to serine 643 in the rat p130cas RLPSP motif responsible for binding the Src SH3 domain. Prior studies have demonstrated serine phosphorylation of proline-rich motifs as a mechanism by which SH3 domain binding is regulated (28). To assess whether human p130cas Ser-639 phosphorylation alters binding to the Src SH3 domain, we carried out pull-down assays in MCF-7 and BCAR3/MCF-7 cells transiently transfected with murine p130cas and RPLPSPP motif responsible for binding the Src SH3 domain. Prior studies have demonstrated serine phosphorylation of proline-rich motifs as a mechanism by which SH3 domain binding is regulated (28). This study, we used cell lysates prepared from MCF-7 and BCAR3/MCF-7 cells transfected with wild type or RLGS mutant p130cas to perform pull-down assays using GST SH3 protein domains from Src, p130cas, or p85α. BCAR3 expression induced binding of GST-Src SH3 but not GST-p130cas SH3 or GST-p85α SH3 to p130cas in an RLP motif-specific manner (Fig. 2A).

In order to determine the specificity of the observed BCAR3-mediated enhancement of Src SH3 domain binding to p130cas, we used cell lysates prepared from MCF-7 and BCAR3/MCF-7 cells transfected with wild type or RLGS mutant p130cas to perform pull-down assays using GST SH3 protein domains from Src, p130cas, or p85α. BCAR3 expression induced binding of GST-Src SH3 but not GST-p130cas SH3 or GST-p85α SH3 to p130cas in an RLP motif-specific manner (Fig. 2A).

We next sought to establish whether BCAR3-enhanced p130cas association with the Src SH3 domain was cell type-specific. Pull-downs with GST, GST-Src SH2, or GST-Src SH3 were performed in COS-7 fibroblast cells transfected with wild type or RLGS mutant p130cas. The results demonstrated that in COS-7 cells, as with MCF-7 cells, co-expression of BCAR3 (AND-34, the highly conserved murine homolog of BCAR3, was used in these studies) and p130cas resulted in association of GST-Src SH3 with the p130cas-BCAR3 complex in an RLPPLPSPP motif-specific manner (Fig. 2A).
BCAR3 Regulates Src SH3 Domain Binding to p130\textsuperscript{cas}

motif-specific manner (Fig. 2B). Of note, overexpression of BCAR3 in COS-7 cells failed to induce a change in p130\textsuperscript{cas} PAGE migration that was comparable with that observed in MCF-7 cells. These results suggest that the BCAR3-induced post-translational modifications of p130\textsuperscript{cas} responsible for reduced electrophoretic migration are not required for BCAR3-enhanced association of the Src SH3 domain with p130\textsuperscript{cas}.

Among NSP Family Members, BCAR3 Preferentially Augments Src SH3 Domain Binding to p130\textsuperscript{cas}—Each of the three human NSP family members are capable of constitutively binding to the carboxyl terminus of p130\textsuperscript{cas}, suggesting that each might be capable of inducing a complex-dependent conformational change in p130\textsuperscript{cas} that could augment Src SH3 domain binding. Among NSP family members, only BCAR3/NSP2 induces significant anti-estrogen resistance and cyclin D1 promoter activation (21). To establish the specificity of the BCAR3 ability to augment Src SH3 binding to p130\textsuperscript{cas}, we performed pull-down assays on MCF-7 cells stably expressing HA epitope-tagged forms of each of the NSP family members. Among the three NSP family members, BCAR3 preferentially induced association of the Src SH3 domain with endogenous p130\textsuperscript{cas} (Fig. 2C, left three lanes). Following transient transfection of each of these NSP cell lines with HA-p130\textsuperscript{cas}, a comparable pull-down detected association of GST-Src SH3 with HA-p130\textsuperscript{cas} in BCAR3-expressing cells but comparatively less binding in NSP3 and none in NSP1-expressing cells (Fig. 2C, three right lanes).

BCAR3-induced Augmentation of Src SH3 Domain Binding to p130\textsuperscript{cas} Is BCAR3-p130\textsuperscript{cas} Complex-dependent—In a study examining the association of the murine homolog of human BCAR3, AND-34, with the p130\textsuperscript{cas} family member HEF1, we found that mutation of HEF1 L751D or AND-34 R743A blocked association of these two molecules (Fig. 3C, left) (29). Mutation of the comparable residue in p130\textsuperscript{cas}, L791D, also blocked association of p130\textsuperscript{cas} with AND-34 or BCAR3 (Fig. 3C, right) (22). In order to test the hypothesis that BCAR3-induced Src SH3 domain association with p130\textsuperscript{cas} requires the ability of BCAR3 and p130\textsuperscript{cas} to form a stable complex, we transfected MCF-7 cells with BCAR3 (AND-34 was used in these studies) and either wild type or L791D HA-p130\textsuperscript{cas} (Fig. 3D, right). Overexpression of BCAR3 induced Src SH3 domain binding to only wild type p130\textsuperscript{cas}. Similarly, when MCF-7 cells were transfected with two forms of BCAR3 previously shown not to bind to p130\textsuperscript{cas} (R743A and SH2/PS), no BCAR3-induced binding of the Src SH3 domain to p130\textsuperscript{cas} was observed (Fig. 3D) (15, 24, 29). Of note, despite their inability to bind to the p130\textsuperscript{cas} carboxyl terminus, each of these BCAR3 constructs is known to induce intracellular signaling pathways that change the phosphorylation state of p130\textsuperscript{cas} (26).

The Amino Terminus of BCAR3 Is Required for Induction of Src SH3 Binding to p130\textsuperscript{cas}—Because the data above suggested that BCAR3-dependent Src SH3 domain binding to p130\textsuperscript{cas} required p130\textsuperscript{cas} BCAR3 complex formation, we next sought to determine whether the BCAR3 GEF-like domain by itself could induce Src SH3 domain binding. An amino-terminal deletion construct containing the carboxyl-terminal 281-amino acid GEF-like domain of BCAR3 (AND-34 539 – 820) did not...
augment GST-Src SH3 binding, suggesting that amino-terminal sequences of BCAR3 were also required (Fig. 4B). However, a deletion construct containing the corresponding amino-terminal portion of BCAR3 (AND-34 1–538) was also inactive in this assay. Although a chimera containing the amino terminus of NSP3 and the carboxyl-terminal BCAR3 GEF-like domain failed to induce Src SH3 domain binding to p130<sup>cas</sup>, such binding was observed following transient overexpression of the converse chimera containing the amino terminus of BCAR3 and the carboxyl-terminal GEF-like domain of NSP3 (Fig. 4C). These results suggest that although an NSP family member GEF-like domain is also required, the preferential ability of BCAR3 to induce Src SH3 binding to p130<sup>cas</sup> lies in its amino terminus.

**BCAR3 Regulates Src SH3 Domain Binding to p130<sup>cas</sup>**

**A.**

Agreement GST-Src SH3 binding, suggesting that amino-terminal sequences of BCAR3 were also required (Fig. 4B). However, a deletion construct containing the corresponding amino-terminal portion of BCAR3 (AND-34 1–538) was also inactive in this assay. Although a chimera containing the amino terminus of NSP3 and the carboxyl-terminal BCAR3 GEF-like domain failed to induce Src SH3 domain binding to p130<sup>cas</sup>, such binding was observed following transient overexpression of the converse chimera containing the amino terminus of BCAR3 and the carboxyl-terminal GEF-like domain of NSP3 (Fig. 4C). These results suggest that although an NSP family member GEF-like domain is also required, the preferential ability of BCAR3 to induce Src SH3 binding to p130<sup>cas</sup> lies in its amino terminus.

**BCAR3 Expression Augments p130<sup>cas</sup>/Src Association by Immune Precipitation**—In order to determine whether BCAR3 expression augments association of full-length Src with p130<sup>cas</sup> in intact cells, we performed immunoprecipitation studies. Consistent with the results obtained by Src SH3 pull-down analysis, endogenous Src was readily identified in immunoprecipitates of endogenous p130<sup>cas</sup> in BCAR3-overexpressing MCF-7 cells but not in parental MCF-7 cells. (Fig. 5A). In a second set of experiments, the effect of stable transduction with wild type forms of BCAR3 and p130<sup>cas</sup> serve as the control for both panels.
cells. Although once again BCAR3 expression increased the association of Src with p130<sup>cas</sup> in BCAR3/MCF-7 cells, overexpression of Src had no additional augmenting effect on the level of Src association with p130<sup>cas</sup> in BCAR3/MCF-7/Src cells (Fig. 5B).

**Complex Formation Dependence of BCAR3-mediated p130<sup>cas</sup> Substrate Domain Tyrosine Phosphorylation**—We have previously reported that BCAR3 overexpression in MCF-7 cells induces p130<sup>cas</sup> tyrosine phosphorylation in a Src kinase-dependent manner (26). The results described above suggested
that BCAR3-mediated enhancement of p130\textsuperscript{Cas} substrate domain tyrosine phosphorylation might require BCAR3-p130\textsuperscript{Cas} complex formation in the same manner that BCAR3-associated augmentation of Src SH3 domain binding does. To test this hypothesis, MCF7 cells were transfected with wild type or R743A BCAR3 (AND-34 was used in these studies). Whereas wild type BCAR3 induced substrate domain tyrosine phosphorylation of endogenous MCF-7 p130\textsuperscript{Cas}, R743A BCAR3 did not (Fig. 6, left). Similarly, wild type BCAR3 induced more tyrosine phosphorylation of co-transfected wild type HA-p130\textsuperscript{Cas} than did R743A BCAR3 (Fig. 6, right). These results indicate that in MCF-7 cells, p130\textsuperscript{Cas}-BCAR3 complex formation augments BCAR-dependent p130\textsuperscript{Cas} substrate domain tyrosine phosphorylation. In contrast, as previously reported, the same experiment demonstrates that BCAR3-p130\textsuperscript{Cas} complex formation is not required for the BCAR3-induced shift in p130\textsuperscript{Cas} PAGE migration because this is induced by both wild type Src (SRC/MCF-7 and BCAR3/ SRC/MCF-7), followed by Western analysis for Src association.

**BCAR3 Regulates Src SH3 Domain Binding to p130\textsuperscript{Cas}**

**A.**

| Untreated | EGF |
|----------|-----|
| MCF-7    | +   | +   |
| BCAR3/MCF-7 | +   | +   |

**LYSATE**

![Blot: CAS](image)

![Blot: BCAR3](image)

![Blot: SRC](image)

![Blot: PO\textsubscript{4}-ERK1/2](image)

![Blot: ERK1/2](image)

**CAS-IP**

**B.**

| SRC/MCF-7 | SRC/MCF-7 | SRC/MCF-7 | SRC/MCF-7 |
|-----------|-----------|-----------|-----------|
| BCAR3/MCF-7 | +   | +   |
| BCAR3/MCF-7 | +   | +   |

**LYSATE**

![Blot: CAS](image)

![Blot: BCAR3](image)

![Blot: SRC](image)

**CAS-IP**

**FIGURE 5. Analysis of the effect of BCAR3 expression on p130\textsuperscript{Cas}/Src association in MCF-7 cells by immunoprecipitation.** A, BCAR3 expression enhances endogenous Src association with p130\textsuperscript{Cas} in MCF-7 cells. Endogenous p130\textsuperscript{Cas} was immunoprecipitated from MCF-7 or BCAR3/MCF-7 cells that had been cultured in medium alone or stimulated with EGF (10 ng/ml) for 30 min, followed by Western analysis for endogenous Src association. B, endogenous p130\textsuperscript{Cas} was immunoprecipitated from either parental MCF-7 or BCAR3/MCF-7 cells or the same lines stably transduced with wild type Src (SRC/MCF-7 and BCAR3/SRC/MCF-7), followed by Western analysis for Src association.

**BCAR3, p130\textsuperscript{Cas}, and Src Co-localize in MCF-7 Cells**—To examine the effect of BCAR3 on the intracellular location of p130\textsuperscript{Cas} and Src, we carried out immunofluorescence studies of these three proteins in MCF-7 cells (Fig. 7A). In wild type MCF-7 cells, endogenous p130\textsuperscript{Cas} is detected in a faint punctate
distribution at the cell periphery. In BCAR3/MCF-7 cells, this peripheral punctate pattern of endogenous p130\textsuperscript{cas} is markedly accentuated. In MCF-7 cells stably transduced with a lentiviral construct expressing constitutively active Y528F Src (Src CA), the punctate peripheral expression of p130\textsuperscript{cas} is accentuated but is primarily limited to discrete areas of cell protrusion. Finally, in BCAR3/MCF-7 cells stably transduced with Src CA, p130\textsuperscript{cas} was dramatically redistributed in a concentrated punctate manner to small cellular protrusions that were accentuated relative to cells expressing either BCAR3 or Src alone.

Immunofluorescence studies examining HA-BCAR3 and Src CA distribution demonstrated that both proteins co-localized with p130\textsuperscript{cas} at the periphery of either Src CA-transduced MCF-7 cells or BCAR3/MCF-7 cells (Fig. 7, B and C). Comparable experiments examining the distribution of each protein separately verified that such co-localization was not the result of “bleed-through” of the fluorescent signal (data not shown). Both Src CA and HA-BCAR3 were also detected in novel centrally located structures in cells overexpressing both proteins (see arrows in Fig. 7, A–C). Confocal studies showed that such central HA-BCAR3-, Src CA-, and p130\textsuperscript{cas}-containing structures were located at the ventral surface of the transduced cells, in the same plane as the peripheral punctate complexes (data not shown). Given that Src signaling plays an important role in the development of podosomes and invadopodia associated with adhesion and matrix degradation, we examined whether expression of BCAR3, either alone or in combination with CA-Src, resulted in the aberrant formation of such structures in MCF-7 cells (30). Phalloidin-based immunofluorescence studies did not reveal F-actin-positive podosomes or invadopodia or enhanced F-actin filament formation in BCAR3-, Src CA-, or BCAR3/Src CA-expressing MCF-7 cells (Fig. 7D).

**DISCUSSION**

In this study, we have demonstrated that expression of BCAR3 augments Src binding to the focal adhesion adapter protein p130\textsuperscript{cas}, at least in part through augmentation of Src SH3 domain binding to a previously identified carboxyl-terminal p130\textsuperscript{cas} RPLPSPP motif. BCAR3-mediated augmentation of Src SH3 domain association is dependent upon the formation of a complex between p130\textsuperscript{cas} and BCAR3 and is observed whether examining endogenous or transiently or stably transduced p130\textsuperscript{cas} in fibroblast or epithelial cell lines. Forms of p130\textsuperscript{cas} lacking the intact RPLPSPP motif fail to show BCAR3-mediated augmentation of Src binding to p130\textsuperscript{cas}.

The carboxyl-terminal GEF-like domain of BCAR3 associates with the carboxyl terminus of p130\textsuperscript{cas} as well as the closely related protein HEF1/NEDD9 (HEF1) (15, 27). The RPLPSPP motif (residues 635–641) that binds the Src SH3 domain lies adjacent to the p130\textsuperscript{cas} carboxyl-terminal four-helix bundle FAT domain that binds BCAR3 and other NSP family members. The interaction of this p130\textsuperscript{cas} FAT domain with NSP3 has now been clarified by a crystal structure of the complex by Mace et al. (16). This study demonstrated that the binding interface between NSP3 and p130\textsuperscript{cas} is extensive (1192 A\textsuperscript{2}), resulting in tight association by isothermal calorimetry (30 nM). Further, despite the homology of NSP family members’ carboxyl-terminal domains to the Cdc25 fold of Ras subfamily GTPases, an accompanying crystal structure of BCAR3 in that study demonstrates that changes in this domain common to all
BCAR3 Regulates Src SH3 Domain Binding to p130<sup>cas</sup>

In contrast, the induction of optimal cell motility by BCAR3 was found to be sensitive to diminution of the ability of BCAR3 to form a complex with p130<sup>cas</sup> family members. Thus, the formation of a complex between BCAR3 and p130<sup>cas</sup> family members appears to influence only a restricted subset of the downstream signaling events induced by BCAR3 overexpression.

The GST pull-down and immunoprecipitation experiments carried out in the current study suggest that enhancement of Src SH3 binding to p130<sup>cas</sup> could be a molecular mechanism for the p130<sup>cas</sup> complex formation-dependent activities of BCAR3. In such a model, the binding of BCAR3 to the carboxyl terminus of p130<sup>cas</sup> alters p130<sup>cas</sup> conformation such that it becomes capable of binding the Src SH3 domain. Consistent with this hypothesis, forms of either p130<sup>cas</sup> (L791D) or BCAR3 (R743A and SH2-P5) that are incapable of forming a BCAR3-p130<sup>cas</sup> complex are also incapable of augmenting Src SH3 domain association to p130<sup>cas</sup>.

Among the NSP family member splice isoforms tested thus far, BCAR3 induces substantially more Src SH3 domain binding to p130<sup>cas</sup> than either NSP1 or NSP3. Notably, however, the GEF-like domains of NSP1, NSP2, and NSP3 are quite similar (see alignment in Fig. 6 of Ref. 29), suggesting that the unique ability of BCAR3 to induce Src SH3 binding might involve an additional requirement for other domains of the BCAR3 protein (29). Studies with chimeric proteins verified this alternate hypothesis. A chimera containing the NSP3 amino terminus and the BCAR3 carboxyl-terminal GEF-like domain induced only modest Src SH3 domain binding to p130<sup>cas</sup>, whereas the reverse chimera containing amino-terminal BCAR3 sequence and carboxyl-terminal NSP3 sequence induced Src SH3 domain binding as robustly as wild type BCAR3. The precise BCAR3 amino-terminal sequence responsible for augmented Src SH3 domain binding remains unknown because NSP3 chimeras containing BCAR3-derived SH2 domain or serine/proline-rich domains were unable to induce Src SH3 binding to p130<sup>cas</sup> (data not shown). Together, these data suggest two requirements for the ability of BCAR3 to induce the binding of the Src SH3 domain to p130<sup>cas</sup>: (a) amino-terminal BCAR3-specific sequences and (b) a carboxyl-terminal GEF-like domain that is capable of binding p130<sup>cas</sup> but is not unique to BCAR3 among NSP family members. A plausible but as of yet untested hypothesis is that some of the amino-terminal BCAR3 sequences required to augment Src SH3 domain binding to p130<sup>cas</sup> interact with the Src SH3 domain rather than p130<sup>cas</sup>. In such a model, the binding of BCAR3 to p130<sup>cas</sup> may additionally alter BCAR3 conformation so as to contribute to the stability of a trimolecular Src-BCAR3-p130<sup>cas</sup> complex.

The RPLPSPP motif has been reported to play an important role in a variety of p130<sup>cas</sup>-associated processes, including Src-mediated transformation. Mouse embryonic fibroblasts derived from p130<sup>cas</sup>-deficient mice were reported to be deficient in Src-mediated transformation as well as Src-mediated podosome formation (33). Reconstitution of p130<sup>cas</sup> knock-out mouse embryonic fibroblasts with wild type p130<sup>cas</sup> restored the ability of Src to induce mouse embryonic fibroblast transformation, but reconstitution with RPLPSPP mutant p130<sup>cas</sup> did not (34). An intact p130<sup>cas</sup> RPLPSPP motif was also required for restoration of cell motility but not actin stress fiber formation.
It should be noted, however, that a requirement for the p130\textsuperscript{cas} SBD in augmenting Src-mediated anchorage-independent cell growth has not been observed by all investigators (35, 36). Src and the p130\textsuperscript{cas} RPLPSPP motif have also been reported to enhance association of p130\textsuperscript{cas} with focal adhesions, although Src appeared to play an adapter role in this process because kinase-inactive K295M/Y527F Src was sufficient (37). Similarly designed experiments demonstrated that mutation of the p130\textsuperscript{cas} RPLPSPP motif to RAAASP reduced Src-induced Matrigel invasion, formation of podosome rosette structures, and MMP-2 activity, although the p130\textsuperscript{cas} substrate domain YXXP motifs were also critical for all of these end points (38). Overexpression of the carboxyl-terminal portion of p130\textsuperscript{cas} in combination with Src results in augmented tyrosine phosphorylation of the cytoskeletal proteins paxillin and cortactin in a manner that requires an intact RPLPSPP motif (39).

Schuh et al. (40) have reported that expression of BCAR3 regulates c-Src kinase activity in COS-1 cells, as judged by phosphorylation of transfected cortactin and paxillin. The authors find two distinct pools of p130\textsuperscript{cas} in BT539 cells, a p130\textsuperscript{cas}-Src complex and a p130\textsuperscript{cas}-BCAR3 complex. Despite the absence of a triple BCAR3-p130\textsuperscript{cas}- Src complex, siRNA-mediated depletion of BCAR3 resulted in a reduction in Src activity and p130\textsuperscript{cas} phosphorylation (40). To explain this apparent discrepancy, the authors propose that BCAR3 augments Src activity in such cells by recruiting p130\textsuperscript{cas} to the cell membrane, thereby bringing p130\textsuperscript{cas} into proximity with membrane-associated Src. Although carried out in distinct cell lines, our work is most consistent with an alternate hypothesis. Our data would suggest that binding of BCAR3 to p130\textsuperscript{cas} augments association of Src with p130\textsuperscript{cas} by enhancing Src SH3 domain binding to the p130\textsuperscript{cas} SBD RPLPSPPP motif. Using both GST-Src SH3 domain pull-down analyses and confirmatory immune precipitation experiments, we find that in MCF7 cells, Src binds detectably to p130\textsuperscript{cas} only in the presence of BCAR3.

Expression of BCAR3 induces serine phosphorylation of p130\textsuperscript{cas} in human breast cancer cell lines that can be recognized as a result of reduced migration in low bisacrylamide PAGE (26). In that study, serine 639 phosphorylation within the p130\textsuperscript{cas} SBD RPLPSPP motif was observed. In the current study, neither a phosphomimetic mutation of the corresponding serine in rat p130\textsuperscript{cas} (Ser-643) to glutamic acid nor an alanine mutation reduced the ability of BCAR3 to augment Src SH3 domain binding to p130\textsuperscript{cas}. Interestingly, the Src SH3 domain has been shown to bind to an RPLPALP motif in the p130\textsuperscript{cas} family member Sin, supporting the concept that a serine residue is not required for binding of this motif to the Src SH3 domain (32). It remains possible, however, that the pull-down assay utilized in the current study to examine the effects of this serine phosphorylation event is not sensitive to all of the binding constraints that would be observed in the tricomolecular complex of all three full-length molecules.

In keeping with the pull-down and immunoprecipitation experiments described above, our immunofluorescence studies demonstrate that BCAR3, p130\textsuperscript{cas}, and Src co-localize in cells overexpressing both CA-Src and HA-BCAR3. Although the co-expression of all three molecules led to the novel appearance of central ventrally located complexes of the three proteins, these novel structures did not fulfill criteria for podosomes or invadapodia, as judged by lack of F-actin staining.

Integrin and FAK signaling resulting in Src-mediated p130\textsuperscript{cas} substrate domain tyrosine phosphorylation has been called a “molecular switch” for cell migration (5). BCAR3 expression also enhances cell motility, and such enhanced cell motility is at least in part BCAR3-p130\textsuperscript{cas} complex formation-dependent (22–24). The current work demonstrates that in MCF-7 cells, BCAR3 enhances both Src SH3 domain association with the p130\textsuperscript{cas} SBD and Src-dependent p130\textsuperscript{cas} substrate domain tyrosine phosphorylation in a BCAR3-p130\textsuperscript{cas} complex-dependent manner. The target or targets of the BCAR3 SH2 domain, however, remain uncertain, and it is likely that identification of the ligands for this domain will further clarify the role of BCAR3 in normal and malignant cell biology.

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