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The GDP Exchange Factor AND-34 Is Expressed in B Cells, Associates With HEF1, and Activates Cdc42

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AND-34, a novel GDP exchange factor, is expressed constitutively at significant levels in murine splenic B cells, but not in murine splenic T cells or thymocytes. In B cell lines, anti-IgM treatment up-regulates AND-34 transcript levels. B cell AND-34 associates with both the docking molecules p130Cas and HEF1 previously implicated in apoptotic, adhesion, and cell cycle-regulated signaling. Overexpression of AND-34 in murine B cell lines activates the Rac family GTPase Cdc42, but not Rac, Rho, RalA, or Rap1. Consistent with this, a subpopulation of AND-34 overexpressing B cells have long filamentous actin-containing cellular extensions. AND-34 overexpression augments both autophosphorylation and kinase activity of the Cdc42/Rac-responsive serine/threonine kinase PAK1. As previously reported for lymphoid cells transfected with constitutively active Cdc42, AND-34 overexpression inhibits SDF-1-induced B cell polarization. These studies suggest that p130Cas and HEF1-associated AND-34 may regulate B cell adhesion and motility through a Cdc42-mediated signaling pathway.

Transient transfection with epitope-tagged deletion constructs for AND-34 and p130Cas have demonstrated that the C-terminal GEF domain of AND-34 associates with the C-terminal region of p130Cas (19). Association of AND-34 with HEF1 has not been assessed.

The human homolog of AND-34, BCAR3, was identified by Dorssers and colleagues (20) as a gene whose overexpression results in conversion of anti-estrogen-sensitive breast cancer cell lines to anti-estrogen resistance. Remarkably, a similar genetic analysis by the same group has more recently identified p130Cas as second gene whose overexpression confers anti-estrogen insensitivity (21). Although the physical interaction of BCAR3 and p130Cas has not been assessed in human cells, these studies suggest that these two proteins may function in a signaling pathway that confers independence from estrogen in human breast cancer. BCAR3 (NSP2) is a member of a family of homologous human genes, NSP1, NSP2, and NSP3 (22). NSP1 has also been shown to bind to p130Cas, as has the murine homolog of NSP3, CHAT (23).

Focal adhesion complexes are dynamic cellular structures that undergo disassembly during cellular detachment, mitosis, and apoptosis. Interestingly, both p130Cas and HEF1 have also been shown to undergo regulated proteolysis during these events. During mitosis, a relatively stable N-terminal 55-kDa form of HEF1 is generated, a process that is abrogated upon mutation of a candidate caspase cleavage site at aa 360 to 363 from DLVD to DLVA (24). Remarkably, HEF1’s N-terminal 55-kDa fragment remains relatively stable during this process and associates with the mitotic spindle (24). During mitosis, less stable C-terminal 65- and 28-kDa fragments of HEF1 are also generated, the latter (p28) thought to be the result of caspase cleavage at a conserved DDDY site at aa 627 to 630 (25). p55, p65, and p28 can also be detected during cellular apoptosis (25). Overexpression of HEF1 in MCF-7 or HeLa cells induces apoptosis, and the death-promoting activity of HEF1 has been mapped to p28 (25). p130Cas has also been shown to undergo caspase-mediated proteolysis during apoptosis (26, 27).

Finally, HEF1 undergoes proteolytic cleavage, generating at least the p28 fragment, in response to cellular detachment, a process that is prevented by integrin receptor ligation (28). The p28 HEF1 fragment formed following cellular detachment is itself capable of inducing cellular rounding when overexpressed (28). Studies of the C termini of p130Cas and HEF1 suggest that this region of these proteins is also important for their association with focal adhesions (28, 29).

In this study, we have investigated the expression and function of AND-34 in the B lineage. We find that AND-34 is expressed in B lymphocytes and that its expression is regulated by signaling through the B cell receptor (BCR). AND-34 binds to B cell HEF1 and to the HEF1 p28 fragment by its GEF domain. Unexpectedly, we observed activation of Cdc42 in B cells overexpressing AND-34, as well as PAK1 autophosphorylation and inhibition of stromal cell-derived factor (SDF)-1α-induced B cell polarization. Our work suggests that AND-34 may join the list of P130Cas and HEF1-associated proteins regulating lymphoid cell motility and adhesion.

Materials and Methods

Cell culture

The murine B cell lines WEHI-231 and Balb17 were used to assess endogenuous AND-34. Mouse primary splenic B cells and T cells were prepared from Balb/c mice using negative selection with commercial available magnetic beads (Miltenyi Biotec, Auburn, CA). Mouse thymocytes were obtained by gentle mechanical dissociation of thymus from young mice. The purity of the cell populations obtained was verified by flow cytometry using fluorescent-conjugated Abs to CD3 and CD19 (BD Pharmingen, San Diego, CA). S194 cells were a gift from Dr. B. Nikolajczyk (Department of Medicine, Boston Medical Center, Boston University School of Medicine, Boston, MA). B cell lines were maintained in RPMI 1640 medium containing 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μM 2-ME. When grown in serum-free conditions, cells were maintained in X-VIVO 20 medium (BioWhittaker, Walkersville, MD). Mouse embryonic kidney (HEK) 293 cells, a mesenchymal HEK cell line, were used to perform the domain association analysis of AND-34 and HEF1.

Antibodies

The following Abs were used in this study: mouse anti-HA (Covance, Richardson, CA), mouse anti-p130Cas (Transduction Laboratories, Lexington, KY), mouse anti-α-tubulin (clone B-5-1-2; Sigma-Aldrich, St. Louis, MO), rabbit anti-myc (Upstate Biotechnology, Lake Placid, NY), mouse anti-α-tubulin, and anti-Cdc42, anti-RalA, and anti-Rap-1 (Transduction Laboratories), rabbit anti-Rho (Upstate Biotechnology), rabbit anti-PAK1 (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-phospho-PAK1 Ser199/204 (Cell Signaling, Beverly, MA). Both the polyclonal rabbit anti-AND-34 and anti-HEF1 Abs have been previously described (18, 24).

Plasmid constructs

The generation of full-length, hemagglutinin (HA)-tagged HEF1 has been previously described (31). pcDNA3-HA55 contains the N-terminal fragment (363 aa) of HEF1 with an HA tag at its N terminus (25). PSRR-65 contains the C-terminal portion of HEF1 (aa 351 to 835) which can be detected by the anti-p130Cas Ab used in this study (25). The p28-myc-His construct contains the HEF1 C terminus (aa 626 to 835) which can be detected by anti-myc (25). The full-length HA-AND-34, myc-AND-34, and AND-34 deletion mutants ΔSH2/Pro, A_GE, and ΔSH2/750 have been previously described (19). The full-length p130Cas construct was a kind gift from Drs. T. Nakamoto and H. Hirai (University of Tokyo, Tokyo, Japan) (32). The p130Cas truncation mutants 775–986 and 833–968 were generated by PCR using full-length p130Cas as a template. The PCR products were cloned into a previously described pcDNA1 vector that included a Kozak consensus sequence, a start codon, and a 12-aa HA epitope (33).

AND-34/IRE5/GEF murine stem cell virus (MSCV) construct and retroviral transduction

Murine HA-AND-34 was subcloned by PCR into the Xho and R1 sites of the retroviral vector pMSCV-IRE5-GFP, a construct that contains a multiple cloning site followed by an internal ribosome entry site sequence and hGFP. The following oligonucleotides were utilized: 5′ MSCVXhoGAG CTGAGTTTCACTGGCCTTACCCCTACG and 3′ MSCVRTGAA TTCGCAACCTGGCCTTAC. The PCR resulted in a single band in agarose gels which was subsequently isolated (QuiexII; Qiagen, Valencia, CA), cleaved with Xho and EcoRI, and cloned into pMSCV-IRE5-GFP. Retroviral-mediated gene transfer was performed as previously described (34). BOSC cells were transiently cotransfected with pCLE-Eco packaging plasmid and pMSCV-IRE5-GFP or pMSCV-HA-AND-34-IRE5-GFP (35). The medium was changed at 24 and 48 h after transfection. At 72 h, the medium was collected, filtered through a 0.22-μm filter (Costar, Cambridge, MA), and 1.25 μl of polybrene (American Bioanalytical, Natick, MA) was added. WEHI-231 or S194 cells were cultured for 16–20 h with the retrovirus and polybrene-containing media, followed by culture in fresh media. Cells were utilized for experiments within 2–3 days of infection. Transduction efficiencies were determined by analyzing cells for GFP positivity using a FACScan flow cytometer.

Transient transfection

Plasmids were transfected into Bosc or HEK 293 cells using Fugene agent (Roche Diagnostics, Indianapolis, IN). Briefly, HEK 293 cells were grown to 50% confluence in 6-well cell culture plates. A total of 100 μl FCS-free DMEM medium was mixed with 3 or 6 μl Fugene reagent and left at room temperature for 5 min. Then, 1 or 2 μg of constructs were added into the Fugene solution and maintained at room temperature for additional 15 min before adding the final mixture into cell cultures. Fresh medium was added into the cell culture on the second day and whole-cell lysates were collected 48 h after transfection.

Immunoprecipitation and Western blot analysis

Western blot analysis was performed as previously described (19). Briefly, cells were lysed in Nonident P-40 (NP40) buffer (0.1% NP40, 200 mM NaCl, 50 mM TrisCl (pH 7.4), 1 mM NaVO₄, and protease inhibitors). After centrifugation at 14,000 rpm (relative centrifugal force = 16,000) for
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5 min, the supernatants were collected. The AND-34 or HEF1/p130Cas protein from 250 to 500 μg of whole-cell lysate was immunoprecipitated with 2 μg of anti-PAK1 Ab for 2 h, followed by further incubation with 5 μl of protein A/G agarose beads for an additional 2 h. The beads were washed three times with lysis buffer. Proteins were released from the beads by boiling in protein sample buffer for 5 min and separated on a SDS-PAGE gel. After transfer to a nitrocellulose membrane, Western blots were developed by ECL following the vendor’s protocol (Pierce, Rockford, IL).

**GT-Pase activation assays**

The levels of activated Rap-1, Rac, Cdc42, and Rho were determined by "pull-down" analysis. The technique for the pulldown analysis has been previously described (36). Cells transfected with control retrovirus and cells transfected with the HA-AND34 retrovirus were cultured in serum-free medium for 2–4 h. In all pulldown assays except that for Rho, 12–15 million cells were harvested in lysis buffer (50 mM TrisCl (pH 7.2), 200 mM NaCl, 5 mM MgCl₂, 1% NP40, 10% glycerol, and protease inhibitors). For Rho pulldowns, lysis buffer consisted of 50 mM TrisCl (pH 7.2), 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS, 500 mM NaCl, and 10 mM MgCl₂. Whole-cell lysate was incubated for 2 h with 6 μl of glutathione sepharose 4B beads preassociated with 6 μg GST-PAK for Rac and Cdc42 assay, GST-rtk-kinase-RBD for Rho assay, GST-RalGDS for Rap-1 assay, and GST-RalBP-1 for Ral assay. The GST-PAK-RBD protein construct was built by subcloning aa 70 to 149 of rat PAK1 (National Center for Biotechnology Information accession no. P55465) into the BamHI and Sal I sites of pGEX (Amersham Pharmacia Biotech, Piscataway, NJ) and was a kind gift of Dr. Z. Luo (Section of Endocrinology, Boston Medical Center, Boston University School of Medicine). The GST-Rho-kinase-RBD construct was generously provided by Drs. X. Ren and M. Schwartz (Department of Vascular Biology, Scripps Research Institute, La Jolla, CA). In all pulldown assays except that for Rho, the beads were washed three times with cell lysis buffer and GTP-bound GT-Pases were released from the beads by addition of 1 × protein sample buffer and boiling for 5 min. For Rho pulldowns, wash buffer consisted of 1% Triton X-100, 150 mM NaCl, and 10 mM MgCl₂. The released GT-Pases were then detected by Western blot analysis. For the GT-Pṣ/Y pulldown, before addition of the conjugated beads, whole-cell lysates were treated with 100 μg MTP G.getBytes (Sigma-Aldrich) for 10 min at 30°C.

**PAK1 kinase and autophosphorylation assay**

Control and HA-AND34-transduced WEHI-231 cells were harvested in NP40 lysis buffer. PAK1 was immunoprecipitated from whole-cell lysate with 2 μg of anti-PAK1 Ab for 2 h, followed by further incubation with 5 μl of protein A/G agarose beads for an additional 2 h. The beads were washed twice with NP40 lysis buffer and two times with PAK kinase buffer (25 mM TrisCl (pH 7.4), 50 mM NaCl, 5 mM MgCl₂, and 1 mM DTT). The kinase reaction was initiated by the addition of 1 μg myelin basic protein (MBP) as a substrate followed by addition of 10 μCi of [γ-32P] ATP and 100 μM cold ATP. The reaction was carried out at 30°C for 30 min and was terminated by adding 2 × kinase sample buffer and boiling for 5 min. Proteins were separated in a 12% SDS-PAGE gel and transferred onto a polyvinylidene fluoride membrane (Nikon, Melville, NY), and photographed with a Hamamatsu digital camera (Hamamatsu Photonics, Hamamatsu City, Japan).

**Actin staining**

Retrovirally transduced WEHI-231 cells were stained for F-actin as described (37). 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 then incubated with 5 μL of Alexa594-phalloidin (Molecular Probes, Eugene, OR) for 20 min at room temperature. Cells were washed twice with PBS, examined with a Nikon Diaphot fluorescence microscope (Nikon, Melville, NY), and photographed with a Hamamatsu digital camera.

**Polarization assay**

One million cells were cultured for 1 h on 24-well tissue culture plates (Costar) coated with 20 μg/ml fibronectin. Cells were then treated with 100 ng/ml of SDF-1α (R&D Systems) for 30 min, fixed with 3.7% formaldehyde/PBS, washed once with PBS, counted, and photographed using an inverted light microscope. Eight different fields were counted and the percentage of polarized cells in each field was calculated according to the following formula: % polarized cells = (polarized cell number/total cell number) × 100. Polarization was scored according to the criteria of Wilkinson (38).

**Statistical analysis**

Data are reported as means ± SE. Comparisons between multiple groups were performed using single factor ANOVA and secondary comparisons were performed using the Tukey test. Statistical analysis was performed using the SPSS statistical software package (SPSS, Chicago, IL).

**Results**

**Cross-linking surface (s)IgM up-regulates AND-34 transcript and protein**

As our prior studies demonstrated expression of AND-34 transcript in murine spleen and lymph node, we sought to determine whether AND-34 transcripts were upregulated by physiologic signaling in murine B cell lines (18). In the immature B cell line WEHI-231, cross-linking of slgM increased AND-34 transcript levels 2 h after stimulation (Fig. 1A). Similarly, cross-linking of slgM in the more mature B cell line Bal17 augmented AND-34 transcript levels within 3 h (Fig. 1B). Constitutive AND-34 transcript was detected in both lines and was not altered within the first hour after anti-IgM treatment.

To determine if anti-IgM-induced alterations in B cell AND-34 transcript levels result in comparable changes in immunoreactive AND-34 protein levels, we performed Western analysis with polyclonal anti-AND-34 antiserum. A 95-kDa immunoreactive band was constitutively present in both Bal17 (Fig. 2A, left panel) and WEHI-231 (Fig. 2A, right panel) B cell lines. In both cell lines, stimulation with anti-Ig further upregulated this species within 6 h. We next performed comparable Western analysis on highly purified preparations of thymocytes, splenic B cells, and splenic T cells (>95% pure as judged by immunophenotyping). While an immunoreactive 95-kDa protein was readily detectable in murine splenic B cells, this protein was undetectable in thymocytes and detected only at very low levels in murine splenic T cells (Fig. 2B, left panel). The specificity of this immunoreactive band was verified by demonstrating that addition of an excess of the immunizing peptide to an immunoblot of splenic B cell lysate selectively eliminated this band’s detection by the polyclonal antiserum against AND-34 (Fig. 2B, middle panel).

**B cell AND-34 associates with both p130Cas and HEF1**

In prior studies of epithelial and stromal cells, we found association of AND-34 with the docking protein p130Cas. As B cells are reported to express both p130Cas and HEF1, we next determined whether B cell AND-34 associated with either of these proteins.

**Figure 1.** BCR cross-linking up-regulates AND-34 transcript levels in murine B cell lines. A. Ten micrograms of total RNA from WEHI-231 cells treated with Fab(′)2 goat anti-mouse IgM Ab for the indicated period of time was Northern blotted and hybridized with a 32P-labeled probe specific for AND-34. Loading was assessed by hybridization with a probe for GAPDH. B. Bal17 cells were treated and analyzed as for A. Loading was assessed by visualizing the 28S ribosomal RNA band with ethidium bromide.
Lysates derived from Bal17 cells were immunoprecipitated with anti-AND34 antisera, followed by immunoblotting with either monoclonal anti-p130Cas or polyclonal anti-HEF1 Abs. The mAb used to detect p130Cas was raised against a C-terminal peptide known to be conserved in HEF1, therefore, it is reactive with both proteins. In contrast, the HEF1 Ab was raised against a peptide specific to HEF1, and is not cross-reactive (24). Immunoprecipitates from Bal17 lysates contained both 130- and 115-kDa species reactive with the anti-p130Cas Abs, consistent with the hypothesis that AND-34 associates with both a 130 kD p130Cas species and a 115 kD HEF1 species in this B cell line (Fig. 3A, left lane). The 115-kDa but not the 130-kDa species contained in anti-AND34 immu

To further analyze the interaction of HEF1 with AND-34 in cells that can be transiently transfected with efficiency greater than that possible in Bal17 or WEHI-231 cells, we transfected HEK 293 cells with either full-length or truncation mutants of AND-34 and HEF1 (Fig. 4, A and B). As expected from our results examining the association of AND-34 with endogenous Bal17 HEF1 described above, after transfection with myc-tagged AND-34 and HA-tagged full-length HEF1, myc-tagged AND-34 was detected in anti-HA immunoprecipitates (Fig. 5A). HEF1 is known to be cleaved at a caspase consensus site at mitosis, generating an N-terminal 55-kDa fragment (p55) and a C-terminal 65-kDa fragment (p65; Fig. 4B) (24). After transfection with myc-tagged AND-34 and HA-tagged p55 HEF1, no detectable myc-tagged AND-34 was present in anti-HA immunoprecipitates, despite adequate expression levels of both proteins (Fig. 5, B and C). In contrast, in HEK 293 cells transfected with HA-tagged AND-34 and nonepitope-tagged p65 HEF1, immunoblotting with an anti-p130Cas Ab known to cross-react with an epitope in the C-terminal domain of HEF1 demonstrated that HA-AND-34 immunoprecipitates contained p65, while a control immunoprecipitate did not (Fig. 5D). Thus, in keeping with our prior results with p130Cas, AND-34 appears to associate with the C-terminal 65 kDa of HEF1.

The GEF domain of AND-34 binds to p28 HEF1 and the C-terminal 135 aa of p130Cas

To determine which AND-34 domain associates with HEF1, HEK 293 cells were transfected with p65 along with HA-tagged

FIGURE 2. AND-34 protein levels rise following BCR cross-linking of murine B cell lines. A. Whole-cell lysates of Bal17 cells or WEHI-231 cells were treated with Fab') goat anti-mouse IgM Ab for the indicated period of time, followed by immunoblot analysis with polyclonal anti-AND-34. B. Thymocytes, splenic B cells, and splenic T cells were prepared that were >95% CD3, CD19, or CD3', respectively, followed by immunoblotting of whole-cell lysates for AND-34 (left panel). The specificity of the 95-kDa immunoreactive band was verified by loss of detection when an excess of the immunizing peptide was added to the immunoblot (middle panel). In both these experiments, loading was assessed by subsequently immunoblotting with anti-tubulin. The same splenic B and T cell lysates were also immunoprecipitated with anti-p130Cas, followed by immunoblotting with anti-AND34 antisera (right panel). As a control, B cell lysate was immunoprecipitated with an isotype control Ab (CT).

FIGURE 3. Endogenous AND-34 associates with B cell p130Cas and HEF1. A. Bal17 cell whole-cell lysates were immunoprecipitated with polyclonal anti-AND-34 antisera, followed by immunoblotting with a mAb that recognizes both p130Cas and HEF1 (left lane) or a HEF1-specific polyclonal antisera (+, middle lane). As a control, Bal17 lysates were immunoprecipitated with preimmune serum, followed by immunoblotting for HEF1 (CT, right lane). B. Bal17 cell whole-cell lysate was either run alone (left lane) or immunoprecipitated with sepharose-protein G beads in the presence of anti-p130Cas Ab (+) or an isotype control (CT) (right two lanes), followed by immunoblotting with polyclonal anti-AND-34 antisera. These data are representative of three experiments performed.

FIGURE 4. Linear map of HA or myc epitope-tagged truncation mutants of AND-34, p130Cas, and HEF1. The name of the construct is given in the column on the left. The amino acids encompassed by each mutant are given in parentheses. Binding results are summarized on the right. The amino acid sequence of murine AND-34 is as in GenBank accession no. AF179866. The amino acid sequence of human HEF1 is as in GenBank accession no. L43281. The amino acid sequence of rat p130Cas is as in GenBank accession no. D29766.
AND-34 deletion mutants containing the Src homology (SH2) domain and the adjacent proline-rich region (ΔGEF), the GEF domain and an adjacent amino-terminal region (ΔSH2/Pro), or the proline-rich region and a truncated GEF domain (ΔSH2/750) (Fig. 4A). After immunoprecipitation with anti-HA Ab, only the AND-34 construct containing the full GEF domain in conjunction with a small region of flanking N-terminal sequence (ΔSH2/Pro) was found to associate with the p65 HEF1 peptide (Fig. 5E). Thus, again in keeping with our previous results assessing the association of AND-34 with p130Cas, truncation of as few as 70 aa from the C terminus of the AND-34 GEF domain abrogates its association with p65 HEF1.

Following treatment of human breast carcinoma cell lines with apoptotic stimuli, HEF1 is cleaved by caspases to generate a 28-kDa fragment (p28) encompassing aa 630–834 (25). To determine whether this fragment associates with AND-34, HEK 293 cells were transfected with a HA-tagged construct expressing the GEF domain of AND-34 (ΔSH2/Pro) along with myc-tagged p28 HEF1. Anti-myc immunoprecipitates contained HA-ΔSH2/Pro/AND-34 (Fig. 5F) and anti-HA immunoprecipitates contained myc-tagged p28-HEF1 (Fig. 5G), demonstrating that the C-terminal 28-kDa portion of HEF1 binds to the C-terminal GEF domain of AND-34.

In prior studies, we have demonstrated that AND-34 associates with the C-terminal 330 residues of p130Cas (638–968; numbering according to Sakai and colleagues, Refs. 32 and 39) (19). To delineate further the minimal domain required for association of p130Cas with AND-34, we engineered three further p130Cas truncation mutants (Fig. 4C) containing the C-terminal 193 aa (Cas833), 135 aa (Cas901), or 67 aa residues (Cas901–968). While transfection of the Cas901 construct failed to yield a detectable HA-tagged protein, perhaps because of instability (data not shown), both Cas775 and Cas833 produced HA-tagged proteins of the appropriate size (Fig. 5H, left panel). After transfection of each of these constructs in conjunction with myc-tagged full-length AND-34, anti-myc immunoprecipitates contained the HA-tagged p130Cas truncation mutants, demonstrating that no more than the C-terminal 135 aa of p130Cas are required for association with AND-34 (Fig. 5H, right panel).

AND-34 overexpression in B cells activates endogenous Cdc42

To examine the effect of overexpression of AND-34 in murine B cells, WEHI-231 and S194 cells, a nonsecreting plasmacytoma cell line, were transduced with a MSCV retrovirus that drives expression of both GFP and HA-tagged AND-34 (AND-34 RV). As a control, cells were transduced with the same retrovirus expressing GFP only (CT RV). As judged by flow cytometry analysis, the transduction efficiencies were >95% for CT RV and >75% for AND-34 RV (Fig. 6A) for WEHI-231 cells. The reduced GFP fluorescence observed in AND-34 RV-transduced cells is likely to be due to the insertion of the HA-AND-34 open reading frame 5′ of the GFP open reading frame. S194 cells showed comparable transduction efficiencies by flow cytometry (data not shown). Western blot analysis confirmed expression of HA-AND-34 in AND-34 RV-transduced WEHI-231 cells (Fig. 6B). Hoechst 33342 staining of live cells showed no significant difference in the cell cycle profile of either CT RV or AND-34 RV-transduced cells (data not shown).

We noted that 2.3 ± 0.3% of AND-34 RV-transduced S194 cells cultured on standard tissue culture plastic consistently developed long protrusions extending to greater than one cell diameter. Such protrusions were absent from CT RV-transduced cells (<0.1%; Fig. 6C). Induction of such long protrusions was also observed when AND-34 was overexpressed in HEK 293 cells.3

Cell protrusions were also detected in WEHI-231 cells, although the protrusions were much shorter than the ones observed in S194 cells. As is characteristic of filopodia, F-actin staining by Alexa594-phalloidin demonstrated that both the S194 and WEHI-231 cellular protrusions were rich in F-actin (Fig. 6D). All cells exhibiting these morphologic changes were GFP-positive as determined by fluorescent microscopy.

Since activation of Cdc42, a member of the Rho subfamily of small GTPases, results in formation of F-actin-rich filopodia (4), we performed “pulldown assays” to determine whether AND-34 overexpression activated endogenous Rho family GTPases in WEHI-231 cells. We used a GST-PAK-RBD construct and a GST-Rhotekin construct to selectively isolate the GTP-bound forms of endogenous Cdc42 and Rac, or Rho, respectively. Significantly higher levels of GTP-bound Cdc42 (Fig. 7A, right panel) but not Rac (Fig. 7B, right panel) or Rho (Fig. 7C, right panel) were present in AND-34 RV-transduced WEHI-231 cells than in CT RV-transduced cells. Consistent with these results, AND-34 overexpression augmented levels of GTP-bound Cdc42 but not Rac (Fig. 7F, right panel, and data not shown) in S194 cells as well (Fig. 7F). Of note, levels of GTP-bound Rac were constitutively high in murine B cell lines, reducing the sensitivity of this assay to detect AND-34-mediated Rac activation. When control whole-cell lysates were first incubated with GTP-γ-S, a nonhydrolyzable analog of GTP, for 10 min at 30°C, high levels of GTP-bound GTPases were detected, confirming the ability of the chimeric proteins used in these assays to detect activated GTPases (Fig. 7, A–C, left panel).

We have previously shown that AND-34 can activate RalA and to a lesser extent Rap-1 and R-Ras in epithelial cell lines (19). Ral activation has recently been linked to the formation of filopodia in epithelial cells (5). Pulldown assays in transduced WEHI-231 cell failed to detect any activation of RalA (Fig. 7E, right panel) or Rap-1 (Fig. 7D, right panel) after overexpression of AND-34. Again, treatment of WEHI-231 cells with GTP-γ-S demonstrated the ability of the assay to detect endogenous GTP-bound RalA and Rap-1 (Fig. 7, D and E, left panel). R-Ras was not examined, as
 FIGURE 7. Overexpression of HA-AND-34 induces activation of endogenous Cdc42 in WEHI-231 cells. Right panels, WEHI-231 cells were transduced with AND-34 RV and with CT RV. To measure the level of GTP-bound GTPases, cells were grown in serum-free medium for 2–4 h and whole-cell lysates were incubated with GST-PAK (Cdc42 and Rac), GST-rostekin (Rho), GST-RalGDS (Rap-1), and GST-RalBP1 (Ral), respectively. After isolation of the GST-chimeric proteins the GTP-bound GTPases were eluted from the beads and immunoblotted with their corresponding Abs. To confirm equal expression of GTPases in CT RV- and AND-34 RV-transduced cells, aliquots of the whole-cell lysate were also immunoblotted with the same Abs. Left panels, To verify the integrity of the chimeric pulldown protein extracts, whole-cell lysates of CT RV-transduced WEHI-231 were left untreated or treated with 100 μM GTP-γ-S, followed by pulldown analysis as described above. F, Whole-cell lysates of AND-34 RV and CT RV S194 cells were subjected to pulldown analysis. GTP-bound Cdc42 was detected by immunoblotting with anti-Cdc42 mAb. Equal expression of Cdc42 was confirmed by immunoblotting aliquots of the whole cell lysate with the same Ab. Data are representative of five experiments.

FIGURE 8. Overexpression of HA-AND-34 activates the serine/threonine kinase PAK1. A, PAK1 was immunoprecipitated from CT RV- and AND-34 RV-transduced WEHI-231 cell lysates and was immunoblotted with a mAb specific for PAK1 autophosphorylated at serine 199 or 204. B, The amount of immunoprecipitated PAK1 was assessed by immunoblotting for PAK1. C, Immunoprecipitated PAK1 from CT RV- and AND-34 RV-transduced WEHI-231 was subjected to an in vitro kinase assay using MBP as a substrate. Phosphorylated MBP was detected by autoradiography. D, The amount of immunoprecipitated PAK1 from the in vitro kinase assay was assessed by immunoblotting with PAK1 Ab.
AND-34 overexpression impairs SDF-1α-induced polarization of WEHI-231 and S194 cells. CT RV- and AND-34 RV-transduced WEHI-231 and S194 were allowed to bind to fibronectin-coated surfaces and treated with SDF-1α for 30 min or left untreated. Cells were photographed and counted by phase-contrast microscopy. The percentage of polarized cells was determined as described in Materials and Methods. A, Morphology of CT RV (top panels) and AND-34 RV (bottom panels) transduced WEHI-231 and S194 cells after SDF-1α treatment. Arrows indicate examples of polarized cells. B, Percentage of polarized WEHI-231 (left panels) and S194 (right panels) before and after SDF-1α treatment (**, p < 0.01; *, p < 0.05). The data represent the mean ± SE.

FIGURE 9. AND-34 overexpression impairs SDF-1α-induced polarization of WEHI-231 and S194 cells. CT RV- and AND-34 RV-transduced WEHI-231 and S194 were allowed to bind to fibronectin-coated surfaces and treated with SDF-1α for 30 min or left untreated. Cells were photographed and counted by phase-contrast microscopy. The percentage of polarized cells was determined as described in Materials and Methods. A, Morphology of CT RV (top panels) and AND-34 RV (bottom panels) transduced WEHI-231 and S194 cells after SDF-1α treatment. Arrows indicate examples of polarized cells. B, Percentage of polarized WEHI-231 (left panels) and S194 (right panels) before and after SDF-1α treatment (**, p < 0.01; *, p < 0.05). The data represent the mean ± SE.

B CELL AND-34 ASSOCIATES WITH HEF1 AND ACTIVATES Cdc42

in this lineage activates endogenous Cdc42. The elucidation of the domains by which AND-34 associates with HEF1 is of considerable interest. As was the case for our study of the interaction between AND-34 and p130Cas, the demonstration that AND-34 associates with HEF1 through its GEF domain suggests that HEF1 may regulate the ability of AND-34 to interact with its target GTPases. To our knowledge, no other GEF has been demonstrated to associate with a protein other than its target GTPase by its GEF domain. Nonetheless, a number of studies have demonstrated that analysis of the functional role of scaffold proteins in regulating signal transduction pathways is difficult, as experimental alterations in the stoichiometry of signaling components may yield nonphysiologic results (9). Careful studies with mutants that alter binding but not the catalytic function of AND-34 will be required to determine how p130Cas and HEF1 influence AND-34’s signaling properties.

We find that, as with p130Cas, AND-34 associates with the C-terminal 28 kDa of HEF1 and further delineates the binding region of p130Cas to the terminal 135 aa of this protein. In studies of a distinct but related gene product, CHAT/NSP3, Sakakibara and Hattori (23) reported that while the C terminus of this 78-kDa protein associated predominantly with p130Cas, anti-CHAT antibodies also identified a 115-kDa protein designated CHAT-H in hematopoietic tissues such as spleen, lymph node, and thymus that associated with HEF1. Although CHAT-H was not cloned in this study, its size and high-level constitutive expression in thymus and spleen make it unlikely that it is AND-34. A cDNA for an alternatively spliced transcript of the CHAT/NSP3 locus has been subsequently identified (National Center for Biotechnology Information no. AB043953) that is likely to represent CHAT-H. Transcript for SH2 domain-containing Eph receptor-binding protein 1, a longer form of murine NSP3 that is reported to associate with Eph receptors, is also present in spleen and thymus cDNA samples, although the association of this protein with Cas family members has not been studied (42).

Prior studies have demonstrated that the C terminus of HEF1 may mediate protein-protein interactions and is transformed into a stable peptide with biologic functions distinct from full-length HEF1 by a physiologically regulated proteolytic process. The C-terminal 137 residues of HEF1 (aa 695 to 832) contain a divergent helix-loop-helix domain that allows homodimerization or heterodimerization of HEF1 as judged by a LexA DNA-binding domain/B42-activation domain two-hybrid analysis performed in yeast (43). Thus, binding of AND-34 to this domain could alter homodimerization or heterodimerization with Cas family members or, possibly, with other proteins. The implications of such modulation of HEF1 or p130Cas dimerization remain unclear at present, but it is possible that signaling within the Cas family member complexes is altered by conversion from dimeric to monomeric forms.

Overexpression of p28 HEF1 causes both apoptosis and cellular rounding, although to what extent the low levels of physiologically generated p28 HEF1 peptide regulate apoptotic and adhesion-related events remains to be established (25, 28). We find that AND-34 associates with HEF1 through HEF1’s C terminus, as judged by coimmunoprecipitation of AND-34 with either transfected p65 HEF1 or p28HEF1 peptides. Thus, it will be important to determine whether AND-34 plays a role in mediating the biologic activities previously observed following overexpression of p28. Deletion of the terminal 28 aa of p28 HEF1 abolishes the ability of this peptide to induce apoptosis. Of note, in prior studies of p130Cas, we found that deletion of as few as 78 aa at the C terminus of p130Cas eliminated association with AND-34. As the C termini of p130Cas and HEF1 target these proteins to the focal adhesion complex, it will also be of interest to determine whether AND-34 regulates the intracellular localization of these docking molecules (28, 29).

As AND-34 contains a C-terminal domain with modest homology to Cdc25 (Ras subfamily GEF) but does not contain a Dbl-like (Rho subfamily GEF) domain, it remains to be determined how AND-34 induces Cdc42 activation. Perhaps the most likely hypothesis is that AND-34 activates a Ras subfamily member, whose resultant signaling ultimately further activates a GEF for Cdc42. To our knowledge, no prior studies have reported activation of Ras subfamily GTPases upon overexpression of a Ras subfamily GEF. However, there is ample precedent for Ras subfamily GTPase-mediated regulation of Rho subfamily GTPase activation, although the precise identification of the components linking these two families often remains elusive (44–46).

In prior collaborative work, transfection of GST-tagged GT-Pases into 32P-labeled Cos7 cells, followed by isolation of the
GTpase and thin layer chromatography to quantify GTpase-associated GTP and GDP, demonstrated that AND-34 augmented levels of GTP-bound RapA and to a lesser extent, R-Ras and Rap1A (19). Importantly, no AND-34 GEF activity on H-Ras was detected in this study. Using a different technique, the pulldown assay, we see no evidence of AND-34-induced activation of endogenous RapA or Rap1 in lymphoid cell lines. The fact that our current results in B cell lines differ from our prior studies in Cos7 cells could be due to the different lineage of cells studied, decreased sensitivity of the pulldown assay, or the fact that we are examining endogenous GTpase. Nonetheless, our data suggest that if AND-34 activates Cdc42 indirectly by acting as a Ras subfamily GEF, it is likely to do so by acting on a GTpase other than Rap or Rap1. Secondly, AND-34 could activate Cdc42 by acting as an adapter protein rather than a GEF. AND-34 binds to p130Cas by its “GEF” domain, suggesting that it could recruit other proteins to the p130 or HEF1 complex by its SH2 domain. Finally, as the homology of the AND-34 C-terminal domain with the Cdc25 domain is only modest (18% identity, 30% homology), it remains possible that AND-34 could directly catalyze Cdc42 GDP exchange. Our future experiments will focus on an in vitro assessment of AND-34’s GEF activity on Ras and Rho subfamily GTpases.

Given that overexpressed AND-34 activates lymphoid Cdc42, what downstream signaling pathways would such an event influence? A wide variety of Cdc42 effectors have been reported, although the number of physiologic Cdc42 effectors is likely to be more restricted (47, 48). Effectors activated by both Rac and Cdc42 include IQGAP1/2 (49), PAK1 (50), PAK5 (50, 51), mixed lineage kinase 2,3 (52), and mitogen-activated protein/extracellular signal-related kinase kinase kinase 1 (53). Effectors selectively activated by Cdc42 include Wiskott-Aldrich syndrome protein (WASP) (54) and N-WASP (55), activated Cdc42-associated kinase (56), and myotonic dystrophy kinase-related Cdc42-binding kinase (58). We find AND-34 overexpression results in activation of PAK1, a serine/threonine kinase previously implicated in cell polarization, motility, proliferation, and the formation of lamellipodia (59, 60).

However, the long cellular extensions we observed in a small percentage of AND-34-overexpressing B cells may result from other Cdc42-activated effectors. In fibroblasts and endothelial cells, PAK4 has been implicated in Cdc42-mediated filopodia formation (56). In Cos7 cells, N-WASP, a ubiquitously expressed WASP-related protein, was reported to be required for Cdc42-induced filopodium formation, while WASP itself was not (61). N-WASP binds to phosphatidylinositol (4, 5) bisphosphate and Cdc42 by its pleckstrin homology and Cdc42-Rac interaction and binding domains, respectively, freeing its C terminus to interact with the Arp2/3 complex, a group of seven proteins which can nucleate actin filaments in vitro (62). Despite these results in Cos7 cells, WASP itself clearly plays a role in leukocyte cytoskeletal regulation as WASP-deficient leukocytes, including B cells, have abnormal cytoarchitecture, polarization, and migration despite the presence of N-WASP (63).

Lymphoid cells stimulated to undergo chemotaxis initially undergo cell polarization, a process in which lamellipodia form at the leading edge and a uropod forms at the rear of the cell, the latter containing augmented levels of the adhesion molecules ICAM-1, ICAM-3, CD43, and CD44 (64, 65). Cdc42 is well known to regulate such leukocyte polarization and cell motility (1, 66). A variety of cell stimuli enhance B lymphocyte motility and cell polarization including the chemokine SDF-1α, a ligand for the CXCR4 receptor (67, 68). Interestingly, while transfection of T cell lines with constitutively active forms of Cdc42, Rac, RhoA, or the Rac GEF, Tiam-1, impairs such polarization, only the dominant-negative form of Cdc42 impaired subsequent SDF-1α-induced T cell chemotaxis (41). Thus, our observation of inhibition of SDF-1α-induced B cell polarization by AND-34 overexpression is consistent with prior studies in which Cdc42 signaling is constitutively activated in lymphocytes. Development of dominant-negative forms of AND-34 will be required to characterize AND-34’s specific role in normal lymphoid cell polarization and motility.

Given that AND-34’s GEF domain binds to p130Cas and HEF1, it is plausible that physiologic signaling in B cells activates AND-34 by inducing release of AND-34 from the Cas family member, perhaps by phosphorylation. Of note, adhesion of Cos cells to fibronectin induced phosphorylation of NSP1, a human protein related to AND-34, as well as a transient reduction in its association with p130Cas (22). Our future studies will focus on identifying the B cell signals that regulate AND-34’s association with these adapter molecules as well as the mechanism by which AND-34 activates Cdc42.

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