p130Cas Regulates the Activity of AND-34, a Novel Ral, Rap1, and R-Ras Guanine Nucleotide Exchange Factor*

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The Ras superfamily of GTPases are 20–30-kDa guanine-nucleotide binding proteins that play a central role in the regulation of cell proliferation, differentiation, and activation (1). The superfamily can be divided into many subfamilies including Ras, Rho, Rap, Arf, and Ran. All members of the superfamily cycle between inactive GDP-bound and active GTP-bound states. Guanine nucleotide exchange factors (GEFs)1 with specificity for different Ras family members induce the dissociation of GDP, allowing the more abundant GTP to bind and activate these GTPases. In an effort to identify genes transcriptionally up-regulated following the induction of thymocyte apoptosis by cross-linking CD3e in vivo, we recently cloned a novel murine cDNA, AND-34, which contains a carboxyl-terminal domain similar to GEFs for one or more GTPases of the Ras subfamily of GTPases, including Ras, Rap, R-Ras, and TC21 (2). In contrast to previously reported Ras subfamily GEFs, AND-34 contained an SH2 domain at its amino terminus.

We also observed that AND-34 forms a complex in cells with p130Cas, a known c-Src substrate and a docking protein that binds to the Crk adaptor protein, focal adhesion kinase, PYK2/RACK1 tyrosine kinases, and PTP-PEST or PTP1B phosphatases (3). Consistent with such interactions, AND-34 undergoes tyrosine phosphorylation following adhesion of trypsinized fibroblasts to fibronectin-coated plates (2). AND-34 is the murine homologue of NSP-2, a CDNA characterized as one of three closely related family members referred to as NSP1, NSP-2, and NSP-3 (4). NSP-1 was also shown to be tyrosine-phosphorylated following treatment of cells with EGF or insulin.

The human homologue of AND-34 was detected in a study designed to detect CDNAs whose expression converts tamoxifen-sensitive breast cancer cells to tamoxifen insensitivity (5). Remarkably, the same screen has recently detected p130Cas (6), adding support to the notion that p130Cas and AND-34 function in a common signaling pathway to regulate hormone-dependent proliferation of breast cancer cells. Additional support for the relevance of these findings is that AND-34 expression correlates with loss of estrogen receptors in hormone-dependent proliferation of breast cancer cells (5). Furthermore, high levels of p130Cas in primary human breast carcinomas are associated with poor relapse-free survival, poor overall survival, and a reduced response rate to tamoxifen in patients with recurrent disease (7).

In the study described below, we have shown that AND-34 is an activator of Rap and to a lesser extent R-Ras and Rap1A GTPases. Our mapping studies and our in vivo GDP exchange assays suggest that p130Cas inhibits the GEF activity of AND-34.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—HEK293 human embryonic kidney cells, a gift from Dr. Barbara Slack (Boston University School of Medicine) (8), were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM Heps, pH 7.4, 10 μM 2-mercaptoethanol. 293 cells were trypsinized 1 day prior to transfection and transfected to 6-well (35 mm) plates. Cells were 80% confluent on the day of transfection. For each well of the plate, 2.5 μg of plasmid DNA was mixed with 5 μl of DNA PLUS reagent (Life Technologies, Inc.) in 125 μl of serum-free DMEM at room temperature for 15 min. 7.5 μl of LipofectAMINE (Life Technologies, Inc.) in 125 μl of serum-free DMEM was then added for a further 15 min. The 250-μl final mixture was then added evenly to cells covered with 0.6 ml of fresh DMEM with 10% FBS and incubated at 37 °C, 5% CO2, for 3 h. One ml of DMEM with 10% FBS was then added to each well.

Immunoprecipitation and Immunoblotting—24 h after transfection, cells were washed with PBS and lysed in ice for 1 h with 1 ml of lysis...
buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EGTA, 1.5 mM MgCl₂, 20 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 μg/ml pepstatin). The protein supernatant was collected after centrifugation at 14,000 rpm (relative centrifugal force = 14,000) at 4 °C for 10 min. 0.5 mg of protein was mixed with the primary antibody for 2 h at 4 °C. 25 μl of protein/A/G-agarose beads (Santa Cruz Biotechnology) were added and incubated overnight at 4 °C. The beads were washed three times with 1 ml of lysis buffer, and protein was released from beads by boiling them with 20 μl of 2× sample buffer for 5 min. The supernatant was run on SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane, and the immunoblot was developed by ECL as described previously (2).

Measurement of GEF Activity in Vivo—Cos7 cells (5 × 10⁵ per 60-mm dish on the day prior to transfection) were transfected by the DEAE-dextran method with expression vectors containing GST-GTPases, GEFs, and/or full-length or SBD-deleted p130Cas, as described in the figure legends. 48 h later, cells were labeled for 4 h in phosphate and serum-free media with 0.05 mCi/ml of ³²P (ICN). GST-fused GTPases were collected by incubation of cell lysates with glutathione-agarose beads (Sigma) for 1 h and washed with lysis buffer. GTPase-bound ³²P-labeled guanine nucleotides were separated by thin layer chromatography (Andrich) and quantitated by PhosphorImager analysis (Molecular Dynamics) as described previously (9). The data are presented as a ratio of GTP/(GDP + GTP).

AND-34 and p130CIC Deletion Mutants—NH₂-terminal hemagglutinin (HA) epitope-tagged deletion mutants of AND-34 were constructed utilizing a pcDNA1 vector in which a Kozak consensus sequence, a start codon, and a 12-amino acid HA-epitope tag were cloned 5' of a BamHI site (10). Contiguous AND-34 sequences were amplified from the 3'-1 cDNA by PCR with Deep Vent DNA polymerase (New England Biolabs) using oligonucleotide sequences derived from the AND-34 open reading frame with flanking EcoRI sites (5' oligonucleotide) and stop codon/XhoI sites (3' oligonucleotide). The 5' ÐSH2 oligonucleotide sequence was derived from AND-34 bp 1130–1149, 5' ÐSH2/Pro from bp 1617–1638, and 5' GEF from bp 1975–1995. The 3' ÐSH2 oligonucleotide sequences were antisense to AND-34 bp 1149–1130, 3' SH2/Pro antisense to bp 1638–1617, and 3' ÐGEF antisense to bp 1971–1949. BclI/XhoI-digested PCR products were ligated to BamHI/XhoI-digested vector. The p130CIC deletion mutants ÐSH3, ÐSB, and ÐASB, and the p130CIC SBD point mutants RPLP and Y762F were generously provided by Dr. Tetsuya Nakamoto and Dr. Hisamaru Hirai (University of Tokyo) and have been described previously (11).

Four hemagglutinin epitope-tagged p130CIC SBD-binding domain mutants were constructed using an identical cloning strategy to that described above for the AND-34 mutants, with the exception that the 5' oligonucleotides contained a flanking BamHI site. Two 5' oligonucleotides, 5PSBD-L and 5PSBD-S, contained 21 nucleotides distal to nucleotides 1947 and 1943 (residues 553 and 638), respectively, of rat p130CIC (GenBank™ accession number D29766). The two 3' oligonucleotides, 3PSBD-L and 3PSBD-S, contained the reverse complement of 21 nucleotides proximal to nucleotides 2920 and 2706 (residues 968 and 890), respectively, of p130CIC. Sequence encoding a core 253-residue region (residues 638–890) previously shown to be required for SBD binding to p130CIC was amplified with 5PSBD-S and 3PSBD-S oligonucleotides and subcloned to generate SBD-Core (predicted size 27.9 kDa) (11). A 415-residue sequence encoding 84 residues NH₂-terminal and 78 residues COOH-terminal to the core SBD region was amplified with 5PSBD-L and 3PSBD-L and subcloned to generate SBD-Long (residues 553–968, predicted size = 46.8 kDa). Two additional sequences corresponding to the core SBD combined with either flanking NH₂-terminal (NH₂-SBD) or COOH-terminal (SBD-COOH) sequence were also amplified and subcloned using the oligonucleotides described above (predicted sizes of 38.4 and 37.8 kDa, respectively). Myc epitope-tagged AND-34—Full-length AND-34 was amplified by PCR with Deep Vent DNA polymerase using oligonucleotides with flanking XhoI (5' oligonucleotide) and EcoRI (3' oligonucleotide) restriction sites. XhoI/EcoRI-digested PCR product was ligated to similarly digested pcDNA3.1/Myc-His B vector (Invitrogen). Myc-tagged AND-34 deletion mutants were similarly cloned using the same oligonucleotide sequences described for the HA-tagged mutants.

RESULTS

AND-34 Stimulates GDP Release from Ral, Rap1, and R-Ras—The existence of a CDC25-like domain at the COOH terminus of AND-34 suggested that the protein had GEF activity against members of the Ras subfamily of Ras superfamily GTPases. The ability of AND-34 to promote the active GTP-bound state of a variety of Ras subfamily members in cells, including RasH, Rap1A, R-Ras, and RalA was tested by cotransfection of individual GST-GTPase fusion constructs along with AND-34 into Cos cells. After labeling cells with ³²P, the proportion of GTPase-associated with GTP in cells was as determined and quantitated by thin layer chromatography. In these assays, AND-34 increased the proportion of GTP bound RalA in cells from -15% GTP to 30% GTP. In comparison, transfection of the same amount of DNA encoding Ral-GDS, a known GEF for Ral, elevated Ral-GTP levels from only -22% (Fig. 1A). AND-34 was less effective at activating R-Ras, elevating its GTP levels from -15 to -23% (Fig. 1B). AND-34 was not as effective as Ras-GRF1, a known R-Ras GEF, which increased R-Ras GTP levels to -29%. AND-34 also elevated Rap1A GTP levels from 5% GTP to -11% GTP. It was not as effective as the known Rap1A GEF, CRKII, which increased Rap-GTP levels to 22% (Fig. 1C). In contrast to RalA, R-Ras, and Rap1A, RasH was unresponsive to AND-34 expression even though RasH did become activated by the known Ras GEF, SOS, under the same conditions (Fig. 1D). Thus, AND-34 appears to be a multifunctional GEF with the ability to activate RalA, R-Ras, and Rap1A but not Ha-Ras.

Most fully characterized Ras-GEFs isolated to date have a Ras-binding domain that allows them to become activated by GTP-Ras. However, inspection of the amino acid sequence of AND-34 failed to reveal such a binding site. As expected, co-
transfection of a constitutively activated Ras did not increase the GEF activity of AND-34 GEF as it did for Ral-GDS which contains a Ras-binding site. Interestingly, for reasons that are not yet clear, activated Ras appeared to decrease the activity of AND-34.

**Association of the AND-34 Carboxyl Terminus with p130Cas**—We have previously shown that AND-34 associates with p130Cas, a focal adhesion docking protein (2, 12). In order to map the site of interaction between these two proteins, we constructed a series of NH2-terminal hemagglutinin (HA) epitope-tagged deletion mutants of AND-34 (Fig. 2). After transient expression of the mutants in HEK293 cells, anti-HA immunoprecipitates were immunoblotted with anti-p130Cas antibody to detect complexes with endogenous p130Cas. Deletion of the SH2 domain (ΔSH2) and proline-rich domain (ΔSH2/Pro) did not significantly reduce binding of AND-34 to p130Cas (Fig. 3). This result was surprising as the polyproline-rich region of AND-34 contains a motif that matched a consensus (XPpP) for binding to the SH3 domain of p130Cas (2).

In contrast, each of four constructs lacking the GEF domain (SH2, SH2/Pro, ΔGEF, and ΔSH2/GEF) failed to associate with p130Cas (Fig. 3). Immunoblots with anti-HA antibody of the corresponding whole cell lysates demonstrated that the HA-tagged truncation mutants were of appropriate size and were expressed with comparable efficiency (Fig. 3).

To delineate further the region of AND-34 responsible for binding to p130Cas, we utilized the ΔSH2 mutant to construct three further mutants in which 70, 140, or 210 amino acid portions of the GEF domain were removed from the carboxyl terminus of AND-34 (Fig. 2, ΔSH2/GEF750, -680, or -610, respectively). In contrast to wild type AND-34, none of the proteins expressed by these constructs bound to p130Cas, suggesting that at least the terminal 70 amino acids of the AND-34 GEF domain are required for this interaction (Fig. 4). However, an AND-34 mutant containing solely the GEF domain failed to bind to p130Cas (Fig. 4) in cells.

To confirm these findings, we synthesized an independent set of deletion mutants in which the Myc epitope tag was placed on the AND-34 carboxyl terminus (nomenclature identical to that of HA-tagged mutants). Once again, immunoprecipitation of three AND-34 mutants (SH2, SH2/Pro, and ΔSH2/GEF) that lacked the GEF domain failed to associate with p130Cas in these assays (data not shown). In aggregate, these experiments demonstrate that the minimum domain required for AND-34 association with p130Cas in cells includes the GEF domain and at least 70 amino acids amino-terminal to it.

**Both the Src-binding Domain (SBD) and the Flanking C-terminal Region of p130Cas Are Required for Association with AND-34 in Cells**—In order to identify the region of p130Cas required for association with AND-34, we transiently co-transfected a full-length Myc-tagged AND-34 construct in HEK293 cells with HA-tagged deletion mutants of p130Cas (see Fig. 5)
Although all three of these constructs were expressed well in transfected 293 cells, only the constructs containing COOH-terminal flanking sequences along with the SBD-Core were efficiently bound to AND-34 (see SBD-Long and COOH-SBD versus SBD-NH2 in Fig. 7).

Full-length but Not SBD-deleted p130Cas Inhibits AND-34 Ral GEF Activity—The surprising observation that AND-34 requires its GEF domain for p130Cas binding suggests that such binding modulates AND-34 GEF activity. To test this hypothesis, we examined the effect of overexpression of p130Cas on the Ral GEF activity of AND-34 by the previously described in vivo GEF assay (Fig. 8). Transfection of full-length p130Cas blocked the ability of AND-34 to promote Ral-GTP levels in cells. Importantly, overexpression of ΔSBD-p130Cas, which we previously demonstrated fails to bind AND-34, had no effect on AND-34-induced Ral GEF activity. The effect of p130Cas was due to a change in the GEF activity of AND-34 since neither p130Cas nor its AND-34-binding mutant had any effect on Ral GTP levels when AND-34 was not co-transfected (Fig. 8). Immunoblots show that p130Cas had no detectable effect on the expression level of AND-34. These results suggest that p130Cas is a negative regulator of AND-34 GEF activity.

DISCUSSION

In this work, we have identified AND-34 as a novel GDP exchange factor with activity in vivo on RalA, Rap1A, and R-Ras GTPases. The spectrum of GTPases activated by AND-34 is somewhat unusual. Previously identified Ras-GEFs have not been demonstrated to have multiple targets. Moreover, Rap and R-Ras are activated by C3G, and Ras and R-Ras are activated by Ras-GRF1, but no GEF identified to date has been able to activate all three (13–15). Our experiments suggest that AND-34 is most effective against RalA, although this conclusion is not firm since the conditions used involved overexpression of both exchange factor and GTPase. Whether the differences in exchange activity observed reflect properties intrinsic to AND-34 or the contribution of additional cellular components remains to be established. For example, the efficiency with which endogenous AND-34 interacts with these GTPases in cells could be affected dramatically by interacting proteins or by the subcellular localization of the endogenous GEF and GTPases. It is also likely that the AND-34 signaling specificity may vary with cell type and with the mechanism by which AND-34 is activated. All of these issues are clearly worthy of study in the future.

AND-34 is the murine homologue of the human protein, NSP2, one of three structurally related proteins termed NSP1-NSP3 by Lu and colleagues (4). SHEP1 (NSP3) was shown to bind to R-Ras and Rap1A but not RasH or RalA (16). However, its isolated CDC25 domain failed to promote nucleotide exchange on any GTPases in vitro. It is likely that AND-34 is one of a family of GEFs that are regulated by similar upstream signals but activate different sets of GTPases.

A further unusual feature of AND-34 is that, unlike most Ras-GEFs reported on to date, it does not have a detectable Ras-binding site, and its activity is not enhanced by co-expression with activated Ras. In this respect, AND-34 resembles Ras-GPS, a recently described Ras-GEF family that contains a pleckstrin homology domain but no Ras-binding site (17). Interestingly, we actually observed an inhibitory effect of Ras expression on AND-34. Presumably this is through an indirect mechanism mediated by some Ras effector system, possibly as part of a negative regulatory network.

AND-34 and other NSP family members have amino-terminal SH2 domains suggesting that they can be modulated by tyrosine kinase signaling. In fact, EGF and insulin promote the tyrosine phosphorylation of NSP1, whereas integrins promote the tyrosine phosphorylation of NSP1 and AND-34 (2, 4). In addition, the SH2 domain of murine NSP3, SHEP1, binds to a phosphorylated tyrosine motif in the juxtamembrane region of the EphB2 receptor, and NSP-3 is heavily tyrosine-phosphorylated in cells expressing activated EphB2 (4, 16). Whether such signals change the GEF activity of AND-34 remains to be determined.

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**Fig. 4.** Truncation of the AND-34 GEF domain eliminates association with p130Cas. *Lower panels,* HA epitope-tagged ΔSH2 (left), but not ΔSBD truncation mutants in which carboxyl-terminal AND-34 sequences were deleted following residue 610, 680 or 750 (center), binds to endogenous p130Cas in HEK293 cells. The immunoreactive bands that migrate with faster mobility than p130Cas are nonspecific. Myc epitope-tagged ΔSH2 (right), but not a Myc-tagged construct containing only AND-34 GEF sequence binds to endogenous p130Cas. *Upper panels,* Western blotting for the HA-epitope or Myc epitope of the AND-34 truncation mutants demonstrates comparable expression of immunoreactive proteins of the appropriate MW in the WCL utilized for the IP experiments.

**Fig. 5.** Linear maps of HA epitope-tagged mutants of p130Cas. A dotted line represents an internal deletion. A bold arrowhead identifies mutations introduced into a proline motif (PLP converted to LGS) or a tyrosine residue (YDYV converted to FDYV).
Another common feature of some members of this class of proteins is their ability to bind to the c-Src substrate and adaptor protein p130Cas. When we set out to map the domains responsible for the interaction of AND-34 to p130Cas, we suspected that it might be through the SH3 domain of p130Cas, as AND-34 contains a polyproline-rich domain. This region includes the conserved motif XPp+P, a motif previously determined to mediate the association of PTP-PEST, PTP1B, focal adhesion kinase, and C3G with the p130Cas SH3 domain (2).

Alternatively, it was plausible that the amino-terminal SH2 domain of AND-34 bound to the p130Cas substrate domain, a region containing nine YDXP sites that are close to the binding motif of the SH2 domain of v-Crk (3). To our surprise, the AND-34 SH2 domain and the proline-rich region were expendable for binding to p130Cas, whereas the carboxyl terminus containing the GEF domain was not. In fact the nature of the binding between the two proteins may be rather complex, since binding required the entire GEF catalytic domain as well as additional amino-terminal sequence. Although our data do not rule out the possibility that AND-34 binds to p130Cas through an intermediary protein, a recent report demonstrating that murine NSP-3 binds directly to the COOH terminus of p130Cas suggests that this is unlikely (18). Along the same lines, our studies also show that AND-34 binds to the COOH terminus of p130Cas, a region encompassing its Src-binding domain (SBD) and flanking COOH-terminal sequences but not its SH3 domain.

The fact that p130Cas overexpression inhibits AND-34 Ral-GEF activity. Cos cells were transfected with GST-Ral with or without the addition of AND-34, p130Cas and/or ΔSBD (Cas-SBD), a p130Cas mutant in which the Src-binding domain has been deleted. The percentage of GTP-bound Ral in cells was determined as described in Fig. 1. The lower two panels demonstrate expression levels of AND-34 (middle panel) or p130Cas or ΔSBD (bottom panel) in whole cell lysates derived from the transfections utilized in the GEF assays. AND-34 was detected using a polyclonal rabbit anti-AND-34 antisera and p130Cas/ΔSBD using an anti-HA antibody (2).
scaffolding proteins that have first been detected as inhibitors of their binding partners subsequently were shown to function in a positive fashion (19). Thus, p130Cas could conceivably normally function to influence the specificity of AND-34 signaling rather than as a regulator of its catalytic activity.

AND-34 is the murine homologue of human BCAR3, a gene cloned on the basis of its ability to convert tamoxifen-sensitive breast cancer cell lines to tamoxifen resistance (5). This finding implies that signal transduction pathways altered by AND-34 overexpression mediate at least some aspects of this process. Many metastatic estrogen receptor-positive breast cancers are treatable with anti-estrogens such as tamoxifen, perhaps because cell proliferation or survival remains dependent upon estrogen. However, such tumors usually progress to a tamoxifen-resistant (estrogen-independent) state. Unfortunately, little is known about the molecular basis for the development of resistance to tamoxifen therapy. If the model system used to detect BCAR3 (AND-34) is reflective of the development of tamoxifen resistance in breast cancer patients then an understanding of the mechanism of AND-34 (BCAR3) action in this system may be quite important. The identifiable domains in AND-34 suggest at least four mechanisms of action could be involved, via the amino-terminal SH2 domain, the proline-rich region, the p130Cas-binding site, and/or the GEF domain.

In this paper we show that the GEF domain can potentially activateRalA, RalPA, and R-Ras GTPases. Thus, signaling pathways downstream of these GTPases are potential mediators. For example, Ral GTPases are known to interact with multiple signaling molecules including RalB1, a GTPase-activating protein for Rac and CDC42 GTPases; filamin, an actin-binding protein; and phospholipase D (20–24). Of particular interest is our recent discovery that Ral GTPases mediate EGF activation of c-Src (25). Elevated levels of EGF receptor family members and c-Src activity are commonly found in breast cancer samples (26). Furthermore, expression of EGF receptor is associated with a lack of response to hormonal therapy in patients with recurrent breast cancer (27). Rap GTPases can activate B-Raf and mediate ligand-induced cell adhesion, whereas R-Ras can activate phosphatidylinositol 3-kinase and influence inside-out integrin signaling and apoptosis (28–31). R-Ras has also been shown to promote invasion of breast cancer cells (29). Which, if any, of these signaling pathways mediate AND-34 action are presently under investigation.

Remarkably, the same screen that detected BCAR3 (AND-34) as an inducer of tamoxifen resistance also recently found human p130Cas (6). The fact that a random screen of genes detected two cDNAs that code for proteins that naturally bind p130Cas is possible, for instance, that both p130Cas and the GEF domain normally function to influence the specificity of AND-34 signaling (25, 32). Our finding that overexpression of p130Cas suppresses the normal function of p130Cas is to suppress AND-34 GEF activity and so firm conclusions must await additional experiments. It is possible, for instance, that both p130Cas and the GEF domain of AND-34 activate common signaling pathways when overexpressed. One obvious set of candidates are c-Src mediated events, because as we recently showed, Ral-GEFs can activate Src in cells and p130Cas has been shown to participate in c-Src signaling (25, 32).

In conclusion, we have identified a new GEF with novel signaling specificity. It can potentially activate multiple members of the Ras subfamily of GTPases, Ral, R-Ras and Rap. It also appears to be regulated by a well characterized adaptor protein, p130Cas. Finally, understanding the interrelationships between AND-34 and p130Cas may reveal important insights into human oncogenesis.

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