The Ras-extracellular signal-regulated kinase (ERK) cascade is a critical intracellular signaling pathway that regulates growth, survival, and differentiation. Previous work established that Ras-GTP binds to, and facilitates the activation of, the protein kinase Raf-1. Recently, it was demonstrated that the cation diffusion facilitator (CDF) proteins are involved in Ras-ERK signaling by use of a Caenorhabditis elegans genetic screen that identified suppressors of activated Ras. In the current work, we demonstrate that CDF proteins may function downstream of Ras, but upstream of Raf-1 in Xenopus oocytes. We also show that the C. elegans protein CDF-1 and its mammalian homologue ZnT-1 bind to the amino-terminal regulatory portion of Raf-1 and promote the biological and enzymatic activity of Raf-1. Furthermore, we show that Zn\(^{2+}\) inhibits Raf-1 binding to ZnT-1. We propose a model in which CDF protein binding facilitates Raf-1 activation.

The Raf-1 protein kinase plays an important role in signal transduction in eukaryotic cells (1–3). Raf-1 is a member of a multigene family that includes A-Raf and B-Raf. Raf family members regulate cell proliferation and differentiation, and are also involved in the pathogenesis of many forms of human cancer. A recent study found that mutations in B-Raf are present in 96% of human melanomas, highlighting the importance of this gene family (4).

When active, Raf-1 phosphorylates and activates MEK1, a protein threonine and tyrosine kinase that, in turn, phosphorylates and activates the mitogen-activated protein kinase (MAPK) family members ERK1 and -2 (hereafter called ERK) (5–7). Raf-1 activation is a highly complex and incompletely understood process. Although the three-dimensional x-ray crystallographic structure of Raf-1 has not been solved, a widely accepted model is that that amino-terminal portion of Raf-1 folds over the carboxyl-terminal half to inhibit its kinase activity (8). 14-3-3 dimers may stabilize the inactive conformation of Raf-1 by interacting simultaneously with phosphoserine 259 and phosphoserine 621 of inactive Raf-1 (9–13). Inactive Raf-1 is also bound to several heat shock proteins that may prevent the proteasome-mediated degradation of Raf-1 and facilitate its cytoplasmic localization (14, 15).

The Ras family of small GTPases plays a key role in the activation of Raf-1 (16–18). When bound to GTP, Ras binds to two domains on Raf-1, the Ras-binding domain comprising amino acids 51–131, and the cysteine-rich domain (CRD) comprising amino acids 139–184 (19–21). Recently, Morrison’s group demonstrated that protein phosphatase 2A dephosphorylates phosphoserine 259 of Raf-1 to release 14-3-3 and promote Ras-GTP binding to the CRD in growth factor-stimulated cells (22). By binding to Raf-1, Ras also promotes the plasma membrane localization of Raf-1. Therefore, Ras-GTP facilitates Raf-1 translocation and activation. However, Ras-GTP is not sufficient in most cases to fully activate Raf-1. First, several phosphorylation events occur at the plasma membrane, which facilitate activation presumably after Ras-GTP binds to Raf-1 (23, 24). These phosphorylation events include those mediated by p21-activated kinase (PAK) family kinases at serine-338, and Sre family kinases at tyrosine 340 and tyrosine 341 (25–27). These phosphorylation sites are located in the hinge region of Raf-1 that separates the amino-terminal regulatory domain from the carboxyl-terminal kinase domain. Phosphorylation events in the hinge region may promote the dissociation of the inhibitory amino-terminal regulatory domain from the kinase domain. A second reason that Ras-GTP may not fully activate Raf-1 is that Ras-GTP binding to Raf-1 is transient. Indeed, Raf-1 remains at the plasma membrane for several minutes after it dissociates from Ras-GTP. Some investigators suggest that Raf-1 binds to lipids in the plasma membrane, such as phosphatidylserine, via its CRD (28). Alternatively, it is possible that Raf-1 interacts with other plasma membrane proteins besides Ras-GTP.

Recently, we determined that the putative zinc transporter CDF-1 was involved in Ras-mediated signal transduction by use of an invertebrate genetic screen (29). In the current study, we investigated the specific role of CDF-1, and its mammalian homologue ZnT-1, in Ras-Raf-MEK-ERK pathway activation. We found that both CDF-1 and ZnT-1 bind directly to Raf-1 via its regulatory domain, and that CDF proteins are required for Raf-1 activation. We therefore propose that cation diffusion facilitator proteins facilitate Raf-1 translocation and activation.

**MATERIALS AND METHODS**

**Plasmid Construction**—The plasmid pSP64T-Hu-Ras\(^{12}\) encoding constitutively active Ha-Ras was a gift from Dr. Deborah Morrison (NCI, National Institutes of Health, Frederick, MD). The pXen1-GST-R18, pXen1-ZnT-1, and pXen1-CDF-1 plasmids were previously described (29, 30). DNAs encoding full-length Raf-1, amino acid residues 1–320 of Raf-1 (NRaf), and amino acid residues 321–648 of Raf-1 (constitutively active Raf-1 or vRaf) were subcloned into a Xenopus expression plasmid, pXen1. GST-CDF-1 mutant constructs and GST-ZnT-1 mutant constructs were created by PCR amplification of the corresponding regions from full-length cdf-1 and ZnT-1 DNA templates, respectively. The PCR

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1 These abbreviations are used: MEK1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; CRD, cysteine-rich domain; CDF, cation diffusion facilitator; GST, glutathione S-transferase; GVBD, germinal vesicle breakdown.

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products were inserted into pGEX-6P-2 (Amersham Biosciences), a bacterial expression vector containing a tac promoter, downstream and in-frame with the GST moiety.

Expression and Purification of GST-CDF-1 and GST-ZnT-1 Mutant Fusion Proteins—GST constructs were transformed into BL21(DE3)-competent cells (Strategene). The transformed cells were spread onto LB plates containing 50 µg/ml ampicillin and incubated overnight at 37 °C. Colonies were picked and incubated overnight without shaking at 37 °C in 5 ml of liquid LB media. The following morning, the bacterial cultures were diluted 1:100 into fresh liquid LB media and grown to 37 °C to an absorbance of 1.0 (600 nm). Protein synthesis was induced in the transformed cells by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.1 mM. Transformed cells were routinely resuspended in GST buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 0.5 mM DTT) and grown at 37 °C without shaking.

Crude bacterial pellets were resuspended with phosphate-buffered saline containing 1 mg/ml lysozyme and incubated on ice for 15 min. The resulting bacterial Cell lysates were given Triton X-100 to a final concentration of 1% and incubated on ice for 15 min. The lysates were then centrifuged at 12,000 × g for 10 min at 4 °C. The GST fusion proteins in the supernatant were purified with glutathione beads. The purified samples were analyzed by SDS-PAGE, and the gels were stained for protein with Coomassie Brilliant Blue.

Xenopus Oocyte Microinjection and Protein Analysis—To prepare RNA for Xenopus oocyte microinjection, linearized plasmid DNA was purified with phenol/chloroform and then with the SPh+ MESSAGE MACHINE in vitro transcription kit (Ambion). RNA production was verified by gel electrophoresis.

Oocytes were surgically removed from mature female Xenopus laevis and defolliculated by incubation in 1 mg/ml collagenase (Sigma type I) for 3–4 h. Fully grown stage VI oocytes were isolated and allowed to recover at 19 °C in medium containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.82 mM MgSO4, 10 mM HEPES (pH 7.4), 0.33 mM Ca(NO3)2, 4H2O, 0.41 mM CaCl2, 2H2O, 1 g/liter bovine serum albumin, 1 g/liter Ficoll 400, and 10 µg/ml penicillin-streptomycin. Within 18 h of isolation, oocytes were injected with water or 30 ng of RNA encoding full-length Raf-1, NRaf, vRaf, CDF-1, ZnT-1, and GST-R18 in various combinations. Some oocytes were injected with 15 ng of RNA encoding constitutively active Ha-Ras (Ha-Ras12) alone. The injected oocytes were incubated at 19 °C and scored for germinal vesicle breakdown (GVBD) at multiple time points. GVBD was determined by the presence of a white spot on the animal pole of the Xenopus oocytes.

To perform Western blot analysis, oocytes were lysed with Nonidet P-40 lysis buffer, and lysates were cleared by low speed centrifugation. Proteins were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in TBST and incubated overnight at 4 °C with anti-phospho-ERK antibody (Cell Signaling), anti-ERK antibody (Santa Cruz Biotechnology), anti-Raf-1(C-12) antibody (Santa Cruz Biotechnology), anti-Raf-1(H-71) antibody (Santa Cruz Biotechnology), anti-14-3-3 (C-19) antibody (Santa Cruz Biotechnology), anti-FLAG (M2) antibody (Sigma), anti-myc clone 9E10 antibody (Upstate), or anti-phospho-MEK1 Ser-218/222 (Upstate). Bound antibody was visualized with alkaline phosphatase or horseradish peroxidase-conjugated secondary antibody and color-developing agents (ECL, Amersham Biosciences). For co-immunoprecipitation assays, agarose-conjugated anti-myc (9E10) antibody and agarose-conjugated anti-FLAG (M2) antibody were used to pull down ZnT-1 and CDF-1, respectively. Immunoprecipitates were washed four to five times with Nonidet P-40 lysis buffer and analyzed by SDS-PAGE as previously described.

ERK activity assays were performed with a p44/42 MAP Kinase assay kit from Cell Signaling, Inc., according to the manufacturer's instructions. In brief, phospho-ERK immunoprecipitates were obtained from oocyte lysates and incubated with recombinant substrate ERK2 proteins, including 200 µM ATP. Kinase reactions were terminated after a 30-min incubation period, and proteins were separated by SDS-PAGE and analyzed by immunoblotting with an anti-phospho-ERK1 antibody.

Raf-1 kinase assays were performed using an Raf-1 Immunoprecipitation Kinase Cascade assay kit from Upstate Biotechnology according to the manufacturer's protocols. In brief, Raf-1 immunoprecipitates derived from cell lysates were incubated with recombinant substrate MEK1 and ERK2 proteins, including 500 µM ATP and kinase buffer. After 30 min, 1 µl of the supernatant was transferred to the second-stage component mixture of myelin basic protein substrate and γ32P-ATP, and incubated at 30 °C for 10 min. Kinase reactions were stopped by transferring the reaction mixture onto phosphocellulose paper. The bound radioactivity was determined by liquid scintillation counting.

Morpholino Antisense Oligonucleotides—Morpholino antisense oligonucleotides directed against mouse ZnT-1 mRNA and standard control morpholino oligonucleotides were designed by Gene Tools, LLC (Philometh, OR). Each oligonucleotide was produced with a 3′-end fluorescein modification. Morpholino oligonucleotides were introduced into NIH/ST3 cells by use of the Special Delivery System with EPEI according to the manufacturer's protocols (Gene Tools, LLC). After EPEI treatment, cells were observed by fluorescent microscopy. Diffuse fluorescence in the cytosol of treated cells indicated successful cytosolic delivery.

Statistical Analysis—All data are reported as mean ± S.E. Statistical analysis was performed by one-tailed Student t test. A value of p < 0.05 was considered to be statistically significant.

RESULTS

CDF Proteins Act between Ras and Raf-1—We previously showed that CDF proteins positively regulate Ras-mediated signaling in both Xenopus oocytes and Caenorhabditis elegans (29). The core enzymatic components of the Ras-ERK signaling cascade include Ras, Raf-1, MEK, and ERK. To determine the precise site of action of CDF proteins, we analyzed meiotic maturation in Xenopus oocytes stimulated by overexpression of CDF proteins with either constitutively active Ras (Ha-Ras12) or constitutively active Raf-1 (vRaf). Co-injection of ZnT-1 RNA with Ha-Ras12 RNA increased the percent of oocytes that underwent germinal vesicle breakdown (GVBD), a marker of meiotic maturation, compared with oocytes injected with Ha-Ras12 alone. In addition, ZnT-1 potentiated the ability of Ha-Ras12 to induce ERK phosphorylation and ERK enzymatic activity as measured by in vitro kinase assay with recombinant Elk-1 protein used as a substrate (Fig. 1A). In contrast, ZnT-1 did not synergize with vRaf to promote GVBD in oocytes and did not potentiate vRaf-stimulated ERK activation (Fig. 1B). Assuming that Ha-Ras12 and vRaf are maximally active at baseline, one model that is consistent with this data is that ZnT-1 acts downstream of, or parallel to, Ras but upstream of Raf.

CDF-1 is the structural homologue of ZnT-1 in C. elegans (29). We previously showed that ZnT-1 is able to rescue the cell bodies of CDF-1 mutant phenotype, indicating that ZnT-1 is a functional homologue of CDF-1 (29). RNA encoding CDF-1 was co-injected with RNA encoding Ha-Ras12 or vRaf into Xenopus oocytes. CDF-1 overexpression potentiated the ability of Ha-Ras12, but not vRaf, to stimulate GVBD and ERK activation. These results demonstrate that CDF proteins have a conserved function to modulate Ras-mediated signaling (Fig. 1, C and D).

CDF Proteins Interact with Raf-1 and 14-3-3—ZnT-1 is considered to be a zinc exporter because of its ability to reduce the intracellular zinc concentration of mammalian cells (31). ZnT-1 is localized at the plasma membrane and promotes zinc ion efflux from the cytosol (31). Previously, we demonstrated that C. elegans with loss-of-function mutations in the cdf-1 gene are specifically sensitized to zinc toxicity and that overexpression of CDF-1 can rescue zinc ion inhibition of Ras-mediated signaling (29). Therefore, CDF-1 may also function as a zinc exporter.

By inspection of the amino acid sequence of the core members of the Ras-ERK signaling pathway, Raf-1 is a possible target for CDF protein action due to its cysteine-rich domain (CRD), which binds Zn2+ (28). This zinc-binding region lies in the amino-terminal half of Raf-1 that is involved in the auto-inhibition of protein kinase activity (8). Point mutations located in the CRD region increase the biological and enzymatic activity of Raf-1 (32). In addition, UV irradiation induces oxidation of the thiol groups of cysteine residues in the Raf-1 CRD, which leads to the release of zinc ions and Raf-1 protein kinase activation (33). Thus, CDF proteins may regulate Ras signaling by binding to and activating Raf-1.
To evaluate the ability of CDF proteins to bind to Raf-1, we performed a series of co-immunoprecipitation experiments. RNAs encoding ZnT-1 and Raf-1 were co-injected into Xenopus oocytes. Twenty-four hours after the injections, protein lysates were generated and anti-ZnT-1 immunoprecipitates were analyzed by anti-Raf-1 immunoblotting, which showed that ZnT-1 binds to Raf-1. Interestingly, ZnT-1 immunoprecipitates also contained Xenopus 14-3-3 (Fig. 2A). In addition, CDF-1 interacted with endogenous Raf-1 and 14-3-3 in Xenopus oocytes (Fig. 2B). We next examined whether CDF proteins bind to Ras. RNA encoding myc-tagged ZnT-1 or FLAG-tagged CDF-1 was co-injected with RNA encoding Ha-Ras<sup>122</sup> into Xenopus oocytes. Anti-myc or Anti-FLAG immunoprecipitates were analyzed by anti-Ras immunoblotting, and this demonstrated that ZnT-1 and CDF-1 do not bind to either Ha-Ras<sup>122</sup> or wild-type Ha-Ras (Fig. 2C). Co-immunoprecipitation experiments also demonstrated that CDF-1 does not bind to MEK (Fig. 2B).

Raf-1 and 14-3-3 are known to form a complex in vivo. Therefore, it is not clear whether the binding of Raf-1 to CDF proteins was direct or indirect via 14-3-3. We injected RNA encoding GST-R18, a peptide inhibitor of 14-3-3 binding to Raf-1, into Xenopus oocytes and also co-injected the oocytes with RNA encoding FLAG-tagged CDF-1. Anti-FLAG immunoprecipitates were obtained from oocyte lysates and subjected to immunoblot analysis with anti-Raf-1 and anti-14-3-3 primary antibodies. GST-R18 completely eliminated the interaction between CDF-1 and 14-3-3, but affected CDF-1 binding to Raf-1 to a lesser degree (Fig. 2D). These results suggest that 14-3-3 does not mediate the interaction between CDF-1 and Raf-1.

**CDF Proteins Potentiate Raf-1 Activity**—The results de-
scribed above suggest that CDF proteins positively regulate Ras signaling by binding to Raf-1. This binding may play a role in Raf-1 activation. Injection of oocytes with RNA encoding wild-type Raf-1, in contrast to constitutively active vRaf, did not effectively promote GVBD in Xenopus oocytes (Fig. 3A). Similarly, injection of oocytes with RNA encoding ZnT-1 did not promote GVBD. However, co-injection of RNA encoding both wild-type Raf-1 and ZnT-1 resulted in a dramatic increase in GVBD (80–90%) (Fig. 3A).

To determine whether ZnT-1 is required for Raf-1 activation, antisense morpholino oligonucleotides directed against murine ZnT-1 were employed. ZnT-1 morpholino oligonucleotides inhibited ZnT-1 protein synthesis in oocytes injected with RNA encoding ZnT-1 (Fig. 3C). NIH/3T3 cells were treated with control morpholino oligonucleotides or ZnT-1 morpholino oligonucleotides, serum-starved for 24 h, and then stimulated with 10% fetal calf serum for 10 min. ZnT-1 morpholino oligonucleotide treatment did not decrease Raf-1 protein levels in NIH/3T3 cells (Fig. 3D). Raf-1 kinase assays demonstrated that Raf-1 enzymatic activity was inhibited in ZnT-1 morpholino oligonucleotide-treated cells compared with control morpholino oligonucleotide-treated cells (Fig. 3D).

**Carboxyl Termini of CDF Proteins Bind to the Regulatory Domain of Raf-1—** Raf-1 is highly conserved within three distinct regions designated CR1, CR2, and CR3. CR1 consists of two domains, a Ras-binding domain and a cysteine-rich domain (CRD), both of which bind to Ras (1–3). CR2, rich in both serine and threonine, contains several phosphorylation sites. CR1 and

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**Fig. 2.** CDF proteins interact with Raf-1 and 14-3-3. A, ZnT-1 binds to Raf-1 and 14-3-3 in oocytes. Xenopus oocytes were co-injected with RNAs encoding Raf-1 and myc-1 epitope-tagged ZnT-1. After 24 h, oocyte lysates were prepared. ZnT-1 immunoprecipitates, obtained with an anti-myc-1 antibody, were analyzed by anti-Raf-1 (upper panel) and anti-14-3-3 immunoblotting (middle panel). Immunoblots were stripped and re-probed with an anti-myc antibody to assess the amount of ZnT-1 protein in the immunoprecipitates (lower panel). B, CDF-1 binds to Raf-1 and 14-3-3. Xenopus oocytes were injected with RNA encoding FLAG epitope-tagged CDF-1. After 24 h, oocyte lysates were prepared. CDF-1 immunoprecipitates, obtained with an anti-FLAG antibody, were analyzed by anti-Raf-1 (upper panel), anti-14-3-3 (second panel), and anti-MEK immunoblotting (third panel). The levels of CDF-1 in the immunoprecipitates were determined by re-probing immunoblots with an anti-FLAG antibody (lower panel). C, ZnT-1 and CDF-1 do not bind to Ras. Oocytes were injected with RNAs encoding myc-1 epitope-tagged ZnT-1 and Ha-RasV12, or FLAG epitope-tagged CDF-1 and Ha-RasV12. 24 h later, lysates were prepared. ZnT-1 and CDF-1 immunoprecipitates derived from these lysates were analyzed by immunoblotting with an anti-Ha-Ras antibody (upper panel). The immunoblots were re-probed with anti-myc-1 or anti-FLAG antibodies to determine the levels of ZnT-1 or CDF-1 in the immunoprecipitates (lower panel). D, 14-3-3 protein does not mediate CDF-1 binding to Raf-1. Oocytes were injected with RNAs encoding GST-R18 and FLAG epitope-tagged CDF-1. CDF-1 immunoprecipitates, obtained with an anti-FLAG epitope antibody, were examined by anti-Raf-1 (upper panel) and anti-14-3-3 immunoblotting (middle panel). The immunoblot was re-probed with an anti-FLAG antibody to measure the amount of CDF-1 in each immunoprecipitate (lower panel). Notice that the amount of Raf-1 associated with CDF-1 was modestly reduced in the presence of GST-R18 (top panel, lane 3), but 14-3-3 binding to CDF-1 was completely blocked (middle panel, lane 3).
CR2 are located within the amino-terminal half of Raf-1, and they negatively regulate the kinase activity located in CR3. In addition, the CRD binds zinc, and release of zinc ions from this domain results in Raf-1 activation (33). We hypothesized that the CRD of Raf-1 interacts with CDF proteins. We injected ZnT-1 RNA together with RNA encoding the amino-terminal half of Raf-1 (amino acids 1–320, NRaf) into Xenopus oocytes and performed co-immunoprecipitation experiments. NRaf was co-precipitated with ZnT-1 immunocomplexes (Fig. 4A). Furthermore, CDF-1 also co-immunoprecipitated with NRaf, indicating that CDF proteins have a conserved function to regulate Ras signaling by binding to the regulatory region of Raf-1 (Fig. 4B).

CDF proteins display high conservation within six predicted transmembrane domains and have four intracellular portions, including the amino terminus, two intracellular loops and the carboxyl terminus (34) (Fig. 5A). The intracellular loops consist of one very small loop, containing about 20 amino acids, and one larger loop that has a cluster of histidine residues between membrane-spanning domains IV and V. This histidine-rich cluster has been proposed to function as a metal-binding domain. Therefore, it is possible that this region binds to Raf-1. However, there are at least two other possible regions in CDF proteins that may interact with Raf-1, the amino- and carboxyl-terminal segments. To identify the interacting region of CDF proteins, we made GST-CDF-1 mutant constructs encoding the amino-terminal segment, the carboxyl-terminal segment, or the histidine-rich loop of CDF-1. GST fusion proteins were

**Fig. 3. CDF proteins activate Raf-1 activity.** A, ZnT-1 potentiates the ability of Raf-1 to promote GVBD in oocytes. Immature Xenopus oocytes were injected with RNAs encoding wild-type Raf-1, ZnT-1, or both. As a control, water was injected into oocytes instead of RNA. 24 h later, groups of 100–200 oocytes in each injection were scored for GVBD. After that, oocyte lysates were generated and examined for wild-type Raf-1 and ZnT-1 protein levels (blots below bar graphs). Oocyte lysates were also examined for ERK activity by in vitro kinase assay with recombinant Elk-1 protein used as a substrate (blot on right, upper panel). In addition, lysates were examined by anti-phospho-ERK immunoblotting (blot on right, middle panel) and anti-total-ERK immunoblotting (blot on right, lower panel). The bar graphs and immunoblots are representative of three separate experiments. B, Raf-1 kinase activity is increased in oocytes expressing both wild-type Raf-1 and ZnT-1 compared with oocytes expressing wild-type Raf-1 alone. Xenopus oocytes were injected with wild-type Raf-1 or wild-type Raf-1 plus ZnT-1 RNA. After 24 h, protein lysates were obtained and used for Raf-1 in vitro kinase assays. Oocyte lysates were also examined for wild-type Raf-1 protein levels (blot below bar graphs). C, ZnT-1 morpholino oligonucleotides block ZnT-1 protein synthesis. Xenopus oocytes were injected with ZnT-1 morpholino oligonucleotides and RNA encoding carboxyl-terminal FLAG-tagged ZnT-1. As a control, control morpholino oligonucleotides were injected into oocytes instead of ZnT-1 morpholino oligonucleotides. After 24 h, protein lysates were prepared and analyzed by immunoblotting with an anti-FLAG antibody (upper panel). The immunoblot was re-probed with an anti-total-ERK antibody to show equal protein loading in each lane (lower panel). D, ZnT-1 knock-down in fibroblasts inhibits serum-induced Raf-1 kinase activity. NIH/3T3 cells were treated with fluorescein-labeled ZnT-1 morpholino oligonucleotides or control morpholino oligonucleotides. Successful cytosolic delivery of morpholino oligonucleotides was confirmed by fluorescent microscopy that showed cytosolic fluorescence (data not shown). After 24 h, morpholino-treated cells were serum-starved for another 24 h and then stimulated with 10% fetal calf serum for 10 min. Raf-1 protein was immunoprecipitated and in vitro kinase assays were performed. Cell lysates were also examined for wild-type Raf-1 protein levels in each sample (blot below bar graphs). The kinase activity from each cell group was normalized to the kinase activity of control morpholino-treated cells in the absence of serum stimulation. Each column shown in B and D reflects the mean kinase activity ± S.E. of triplicate determinations.
expressed in *Escherichia coli* and purified on glutathione beads (Fig. 5B). The beads were incubated with *Xenopus* oocyte lysates and were washed extensively. The carboxyl-terminal segment of CDF-1 protein, but not the histidine-rich loop, associated with Raf-1 (Fig. 5C). Next, we investigated whether Raf-1 interacts with ZnT-1 in the homologous region. We made GST constructs encoding the histidine-rich loop or carboxyl-terminal segment of ZnT-1. Recombinant GST fusion proteins were expressed in bacteria and purified on glutathione beads (Fig. 5D). GST pull-down assays were performed by incubating beads that contained GST-truncated ZnT-1 fusion proteins with oocyte lysates that contained overexpressed Raf-1. By this method, the carboxyl-terminal segment of ZnT-1, but not the histidine-rich loop, bound to Raf-1 (Fig. 5E). The carboxyl-terminal segment of ZnT-1 also bound to Raf-1 derived from HEK 293 cell lysates (data not shown).

Our results showed that full-length CDF proteins bind to the regulatory region of Raf-1 and that full-length Raf-1 binds to the carboxyl-terminal segment of CDF proteins. To confirm that the carboxyl-terminal segment of CDF proteins could bind directly to the regulatory region of Raf-1, we incubated glutathione beads containing the carboxyl-terminal segment of CDF-1 or ZnT-1 with oocyte lysates that overexpressed NRaf. These experiments confirmed that the carboxyl-terminal segment of ZnT-1 and CDF-1, but not the histidine-rich loop, associated with NRaf (Fig. 5, F and G).

**Zinc Ions Inhibit Raf-1 Binding to ZnT-1**—The results described above show that CDF proteins bind to the amino-terminal half of Raf-1 and potentiate Raf-1 activity. In our previous work, we demonstrated that increasing the concentration of intracellular zinc ions inhibits Ras-mediated signaling in *Xenopus* oocytes and in *C. elegans* (29). We hypothesized that increased intracellular zinc concentration blocks the ability of CDF proteins to bind to Raf-1. To test this hypothesis, oocytes were injected with RNA encoding either ZnT-1 or Raf-1. ZnT-1 immunoprecipitates from injected oocyte lysates were incubated with 150 mM ZnSO$_4$ or CoSO$_4$, and then Raf-1-containing oocyte lysate was added to the immunoprecipitates. Addition of zinc but not cobalt to ZnT-1 blocked its ability to bind to Raf-1 in *vitro* (Fig. 6).

**Extracellular Zinc Blocks ZnT-1-mediated Raf-1 Activation**—ZnT-1 is localized at the plasma membrane and promotes zinc efflux from the cytosol (31). In the experiments described above, oocytes that have a total intracellular zinc concentration of $\sim$1 mM (35), were bathed in a solution lacking added zinc, resulting in a significant cytosolic-to-extracellular fluid gradient. This gradient may promote ZnT-1-mediated zinc efflux from cells. To determine whether zinc efflux is an important aspect of ZnT-1-mediated Raf-1 activation, we performed experiments where the gradient of Zn$^{2+}$ between the cytosol and the extracellular solution was reduced. Oocytes were injected with Raf-1 RNA alone or with Raf-1 RNA and ZnT-1 RNA. Oocytes expressing Raf-1 and ZnT-1 were incubated with the oocyte bathing solution in the presence or absence of 0.1 mM ZnSO$_4$. Adding Zn$^{2+}$ to the oocyte bathing solution blocked the ability of ZnT-1 to promote Raf-1 enzymatic activity as measured by *in vitro* kinase assay with recombinant MEK-1 protein used as a substrate (Fig. 7). Adding Zn$^{2+}$ to the bathing solution did not affect the protein levels of ZnT-1 or Raf-1 (Fig. 7).

**DISCUSSION**

The recent determination that activating mutant forms of B-Raf are found in the majority of malignant melanomas illustrates the importance of Raf family members in human physiology and disease (4). Activation of Raf proteins is a complex, multistep process that is incompletely understood, despite intense investigation for more than two decades (1–3). In this work, we focused on the role of CDF proteins in Raf-1 activation.

We previously identified mutations in CDF-1 that suppressed the activated Ras phenotype in a *C. elegans* genetic screen (29). CDF-1 is a homologue of ZnT-1, a zinc transporter that facilitates the export of Zn$^{2+}$ out of mammalian cells. In our previous work, we demonstrated that overexpression of CDF-1 or ZnT-1 in *Xenopus* oocytes promoted ERK MAPK activation and meiotic maturation (29). We also demonstrated that elevated zinc concentration suppressed ERK MAPK activation in *Xenopus* oocytes (29). Furthermore, we demonstrated that increased dietary zinc was lethal in *C. elegans* that lacked *cdf-1* but that zinc did not harm wild-type worms. Our working model based on our initial work was that CDF proteins promoted ERK activation by lowering cytosolic zinc concentrations, although we did not identify the specific target of zinc.

In the current work, we analyzed the role of CDF proteins in Ras-ERK signaling by use of *Xenopus* oocytes and cultured...
mammalian cells. We demonstrated that CDF proteins could potentiate Ha-RasV12- and wild-type Raf-1-mediated GVBD in oocytes but could not enhance vRaf-mediated GVBD. One model, in which CDF proteins act downstream of Ras but upstream of Raf-1, explains these results.

Although we previously hypothesized that CDF proteins ac-
ZnT-1 were incubated in oocyte bathing solution in the presence or in the absence of Raf-1 and myc-1 epitope-tagged ZnT-1. Oocytes expressing Raf-1 and myc-1 were injected with RNA encoding Raf-1 alone or Raf-1 and myc-1. Oocytes expressing Raf-1 and myc-1 were incubated in oocyte bathing solution in the presence or absence of added 0.1 mM ZnSO₄. After 24 h, oocyte lysates were generated. Raf-1 protein was immunoprecipitated and in vitro kinase assays were performed by use of recombinant MEK-1 protein as a substrate (upper panel). In addition, lysates were examined by anti-myc-1 immunoblotting (middle panel) and anti-Raf-1 immunoblotting (lower panel).

FIG. 6. Zinc ions inhibit the association of Raf-1 and ZnT-1 protein. Xenopus oocytes were injected with RNA encoding myc-1 epitope-tagged ZnT-1. After 24 h, ZnT-1 was immunoprecipitated by use of an anti-myc antibody, incubated with 150 mM ZnSO₄ solution, or water for 1 h at 4 °C, and then an excess of oocyte lysate expressing Raf-1 was added to immunoprecipitated ZnT-1 and incubated for 8 h (final concentration of ZnSO₄ or CoSO₄ was 150 mM). Immunoprecipitates were washed extensively and analyzed by immunoblotting with anti-Raf-1 antibody (upper panel). The immunoblot was re-probed with an anti-myc-1 antibody to determine the amount of ZnT-1 in the immunoprecipitates (lower panel).

FIG. 7. Extracellular zinc blocks ZnT-1-mediated Raf-1 activation. Xenopus oocytes were injected with RNA encoding Raf-1 alone or Raf-1 and myc-1 epitope-tagged ZnT-1. Oocytes expressing Raf-1 and ZnT-1 were incubated in oocyte bathing solution in the presence or absence of added 0.1 mM ZnSO₄. After 24 h, oocyte lysates were generated. Raf-1 protein was immunoprecipitated and in vitro kinase assays were performed by use of recombinant MEK-1 protein as a substrate (upper panel). In addition, lysates were examined by anti-myc-1 immunoblotting (middle panel) and anti-Raf-1 immunoblotting (lower panel).

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