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Abl Family Kinases and Cbl Cooperate with the Nck Adaptor to Modulate *Xenopus* Development*

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We previously showed that overexpression of the Nck Src homology (SH) 2/SH3 adaptor in *Xenopus* embryos induced developmental defects including anterior truncation and mesoderm ventralization. Mutagenic analysis indicated that this was due to relocalization of endogenous proteins that bind the first two SH3 domains of Nck. We therefore screened a *Xenopus* expression library with Nck SH3 domains to identify Nck-interacting proteins, and evaluated candidate binding proteins for a potential role in Nck-induced anterior truncation/ventralization. Of 39 binding proteins analyzed, only the Abl-related kinase Arg and the Cbl proto-oncogene product bound preferentially to the first two SH3 domains in tandem compared with the individual domains, consistent with a role in the developmental phenotype. High level overexpression of c-Abl or Arg alone induced anterior truncation, as did lower levels of an activated form of Abl; Cbl alone had no effect. In a sensitized system where subthreshold amounts of a ventralizing Nck mutant were expressed, co-expression of the combination of Abl or Arg and Cbl at modest levels strongly potentiated anterior truncation, while Arg, Abl, or Cbl alone were without effect. These results suggest a role for both Cbl and Abl family kinases in patterning the *Xenopus* embryo.

Nck is an Src homology (SH)[1] 2/SH3 adaptor protein, which is thought to mediate the formation of multiprotein complexes in signal transduction. It consists of three SH3 domains and an SH2 domain (1), each of which can bind to other proteins *in vivo* and *in vitro* (reviewed in Ref. 2). Little is known about the physiological role of Nck, although the *Drosophila* homolog, Dock, has been shown to be required for proper guidance and targeting of retinal axons (3). Nck has also recently been implicated in modulating the response to T cell receptor engagement (4, 5) and in actin polymerization on the surface of vaccinia virus particles (6).

Earlier work from our laboratory implicated Nck (and Nck-interacting proteins) in mesoderm patterning during early *Xenopus* development. We found that injection of RNA encoding mutant forms of Nck in which the first two SH3 domains were intact into the two dorsal blastomeres of four-cell stage embryos resulted in developmental defects. Injected embryos exhibited anterior truncation, and both whole embryos and activin-treated animal cap explants showed evidence of ventralization, with presumptive dorsal mesoderm adopting a more ventral fate (7). The simplest interpretation of these results is that Nck expression alters the response of presumptive dorsal mesoderm to morphogenetic signals, affecting mesoderm patterning prior to and during gastrulation and thereby leading to defects in the dorsoanterior tissues of the resulting embryo. Mutation of the SH2 domain or either of the first two SH3 domains (SH3-1 and SH3-2) of Nck diminished ventralizing activity, while a construct encoding both domains (SH3-1+2) fused to a membrane-targeting signal promoted ventralization (7). We therefore concluded that relocalization of an endogenous protein or proteins that binds both SH3-1 and SH3-2 to sites of tyrosine phosphorylation on the membrane was responsible for the Nck-induced ventralization/anterior truncation phenotype.

Here we describe a screen for *Xenopus* Nck-binding proteins that may function downstream of Nck in mesoderm patterning. The results of this screen suggest that the Cbl proto-oncogene, and Arg and/or its close relative the Abl proto-oncogene, are likely to play a role in the Nck-induced patterning defects. The Abl and Arg nonreceptor tyrosine kinases are implicated in the control of cell proliferation, as well as neural development and responses to adhesion and DNA damage (Refs. 8 and 9; reviewed in Refs. 10 and 11). Cbl has long been known to downmodulate signals from some tyrosine kinases (12), and has recently been shown to be a component of a ubiquitin ligase complex, targeting its tyrosine-phosphorylated binding partners for internalization and/or degradation (13–16). To our surprise, we find that in *Xenopus* Abl family kinases and Cbl work coordinately to enhance the Nck-induced phenotype. Although previous results had suggested that Nck SH3 domains could bind Abl and Cbl (17–19), these data provide the first

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF237765 and AF237766.

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[1] The abbreviations used are: SH, Src homology; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; aa, amino acid(s); DAI, dorsoanterior index; PDGF, platelet-derived growth factor; PAGE, polyacrylamide gel electrophoresis; wt, wild type; HA, hemagglutinin; FGF, fibroblast growth factor.

evidence for the concerted actions of Abl/Arg, Nck, and Cbl in signal transduction.

EXPERIMENTAL PROCEDURES

Plasmids and Constructs—HA-tagged SH3 domains from human Nck were generated by polymerase chain reaction (PCR) (20) and cloned into pGEX-2N. Glutathione *S*-transferase (GST) fusion proteins were purified by binding to glutathione-agarose beads using standard protocols. Full-length human Cbl cDNA was obtained from H. Band. Internal deletion mutants of Cbl and murine Abl were generated by two-step PCR mutagenesis; primer sequences are available upon request. The *Xenopus* Abl fragment corresponds to amino acids (aa) 110–517 of murine type IV Abl and was kindly provided by K. Dorey and G. Superti-Furga.

Library Construction and Screening—RNA was purified from stage 8 *Xenopus* embryos using Trizol reagent (Life Technologies, Inc.), mRNA isolated by oligo(dT) cellulose chromatography, and cDNA was synthesized using oligo(dT) primers and a cDNA synthesis kit (Stratagene). cDNAs were ligated into the Lambda ZAPII vector and packaged following manufacturer's recommendations (Stratagene). Average insert size ranged from 0.5 to 6 kilobase pairs, analyzed by agarose gel electrophoresis. ~50,000 phage were plated and duplicate nitrocellulose filters obtained. Filters were blocked overnight with 1% ovalbumin in Tris-buffered saline plus Tween 20 (TBST: 150 mM NaCl, 10 mM Tris pH 8.0, 0.05% Tween 20) at 4 °C, then incubated in the same buffer containing GST-Nck SH3-1+2-HA (2 μg/ml) for 2 h at 4 °C. Positive plaques were detected with monoclonal anti-HA antibody (HA-11, BAbCo), followed by goat anti-mouse conjugated alkaline phosphatase (Jackson Immunoresearch). False positives were eliminated by binding to GST and detection with a monoclonal anti-GST antibody (21) (kindly provided by M. Matsuda). cDNAs were excised from positive clones with the ExAssist helper phage (Stratagene). The *Xenopus* Arg and Cbl phage clones correspond to aa 392–end of human type Ib Arg and aa 342–end of human Cbl, respectively. The sequences have been deposited in GenBank™ (accession numbers AF237765 and AF237766).

Nck Binding Assays—Partial cDNAs were transcribed and translated in the presence of [^{35}S]methionine using the TnT Quick Coupled Transcription/Translation System following manufacturer's protocols (Promega). One third of the reaction contents were then incubated with 2.5 μg of GST proteins bound to 5 μl of glutathione-agarose beads in 75 μl of TNGT (20 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100) at 4 °C for 1 h. Beads were washed four times with TNGT, and bound proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Northern Blotting—RNA was prepared from pooled staged embryos using Trizol reagent following manufacturer's instructions. Polyadenylated RNA was isolated by oligo(dT) chromatography using standard protocols. One μg of poly(A)$^+$ RNA/lane was electrophoresed in agarose gels containing formaldehyde and transferred to nylon membranes by capillary action. Filters were hybridized with [α-^{32}P]UTP-labeled riboprobe overnight and washed three times in 0.1× SSPE (1× SSPE: 150 mM NaCl, 10 mM NaH_2PO_4, 1.25 mM EDTA, pH 7.4), 0.5% SDS at 65 °C before exposure to film. Antisense RNA probes were synthesized *in vitro* using T7 or T3 RNA polymerase from linearized pBK (Stratagene) or pGHXP (7) plasmid templates.

Mammalian Cell Transfection and Immunoprecipitation—Human 293T cells were maintained and transfected as described (22) and lysed in 1 ml of TXB (20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1% (v/v) aprotinin solution (Sigma A6279)). For Fig. 3*B*, 300 μl of normalized lysate was precleared with 30 μl of protein A-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C. Supernatants were then incubated with 1 μg of affinity-purified rabbit anti-Nck antibody or 1 μl of crude preimmune serum for 2 h at 4 °C. Immune complexes were collected on 20 μl of protein A beads and washed three times in Tris-buffered saline plus Tween 20 before adding sample buffer, boiling, and subjecting to SDS-PAGE and immunoblotting with monoclonal anti-Abl antibody (8E9, PharMingen). For Fig. 4, 100 μl of lysate was pre-cleared for 1 h with 10 μl of protein G-agarose beads (Pierce) and supernatant incubated with 1 μg of 9E10 anti-Myc monoclonal antibody for 2 h at 4 °C. Immunoprecipitates were collected on 10 μl of protein G-agarose beads and washed twice in TXB, once in TXB with 0.5 M NaCl, once in TXB with 10 mM NaCl, and twice in TXB again before SDS-PAGE and immunoblotting with polyclonal anti-HA antibody.

Xenopus Microinjections—All constructs used to synthesize mRNA for microinjection were in the vector pGHXP, which is derived from pGEM-HE (7). Nck mutants used here have been described (7). Murine type IV c-Abl, human type 1B Arg (provided by G. Kruh), and HA-tagged human c-Cbl were fitted with appropriate restriction sites by PCR and subcloned into pGHXP. After linearization of plasmids with *Nhe*I, capped mRNAs were synthesized by *in vitro* transcription using the mMessage mMachine T7 kit (Ambion, Inc.) following manufacturer's protocols. RNA was quantitated by ethidium bromide staining.

Frogs were maintained and fertilized eggs obtained following standard protocols (23). Embryo staging and quantitation of dorsoanterior index (DAI) were according to Nieuwkoop and Faber (24) and Kao and Elinson (25), respectively.

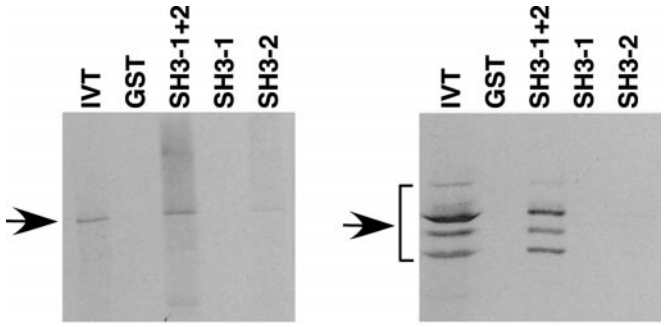

FIG. 1. ***Xenopus* Arg and Cbl bind specifically to Nck SH3-1+2.** Partial *Xenopus* cDNA clones encoding Arg (*left*) or Cbl (*right*) were subjected to *in vitro* transcription and translation, and ^{35}S-labeled translation products were incubated with glutathione-agarose beads bound to GST, GST-Nck SH3-1+2, GST-Nck SH3-1, or GST-SH3-2 as marked. Beads were washed and bound proteins separated by SDS-PAGE and detected by autoradiography. *IVT*, input *in vitro* translation reaction before bead binding. *Arrows* indicate position of Arg and Cbl translation products. For Cbl, multiple Cbl mRNA-dependent bands are observed, presumably due to proteolytic degradation.

RESULTS

Our previous experiments involving expression of Nck mutants in early *Xenopus* embryos showed that Nck constructs in which the first and second SH3 domains (SH3-1 and SH3-2) were intact induced anterior truncation and mesoderm ventralization (7). Because Nck consists only of modular protein-interaction domains, this phenotype must be a consequence of endogenous proteins binding to Nck. Since our prior mutational analysis suggested that both SH3 domains of Nck were required in cis for the mesoderm patterning phenotype (7), we aimed to identify *Xenopus* proteins that preferentially bind to Nck SH3-1+2 compared with either domain alone.

We constructed a λ phage expression library using cDNA from mid-blastula (stage 8) embryos and screened the library for binding to a GST-SH3-1+2 fusion protein. Thirty-nine independent SH3-1+2-binding clones were isolated and further characterized. We tested each of these clones for binding to purified SH3 domains, and only two of these were found to bind more tightly to SH3-1+2 than to the individual SH3 domains. Sequencing revealed that these clones corresponded to the *Xenopus* homologs of the proto-oncogene Cbl and the nonreceptor tyrosine kinase Arg, which is highly related to the Abl proto-oncogene. As shown in Fig. 1, when the cDNA inserts corresponding to *Xenopus* Arg and Cbl were transcribed and translated *in vitro*, the translation products bound very well to the GST fusion containing both SH3-1 and SH3-2, but weakly or not at all to the individual domains. This is consistent with results of another group, which found that multiple SH3 domains were required for mammalian Nck to interact with Cbl or BCR-Abl (17). For all other clones, solution binding was either weak for all domains tested, or required only the second SH3 domain, which seems to dominate interactions relative to SH3-1 (data not shown).

Because the mesoderm patterning defect associated with Nck expression is first manifested in the early gastrula stage (7), any *Xenopus* protein involved in the phenotype would need

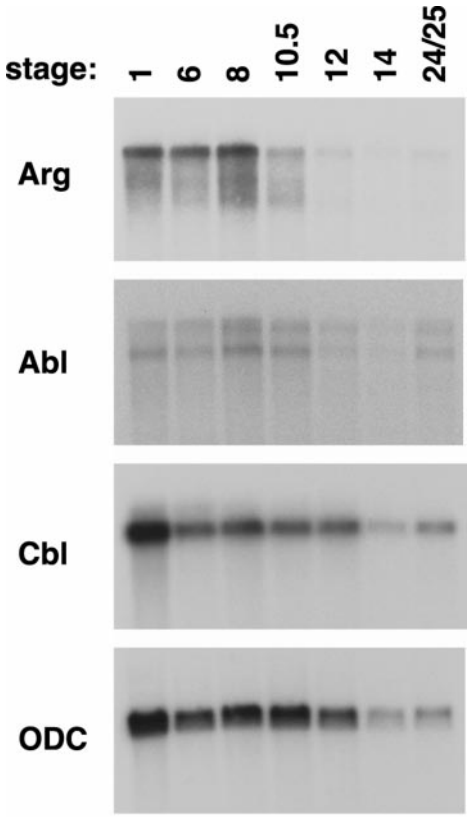

FIG. 2. **Expression pattern of *Xenopus* Arg, Abl, and Cbl.** Poly(A)$^+$ RNA from pooled staged embryos was subjected to Northern blotting using ^{32}P-labeled riboprobes to *Xenopus* Arg, Abl, or Cbl as indicated. Ornithine decarboxylase (*ODC*) probe was used as a loading control.

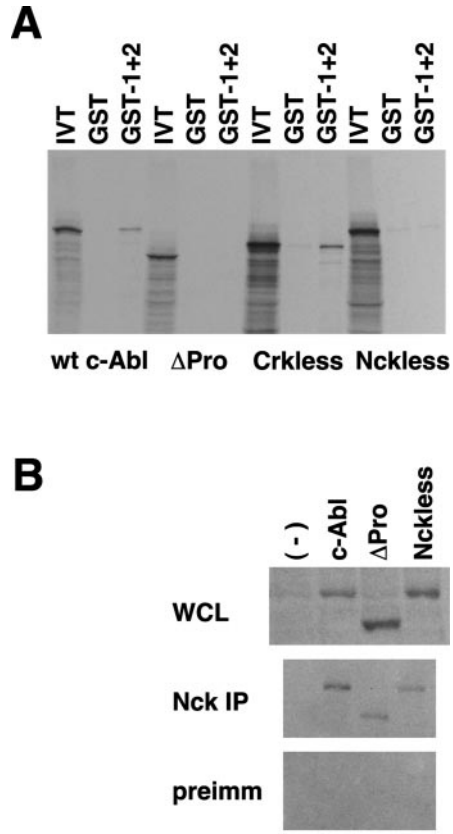

FIG. 3. **Mapping the binding site for Nck SH3-1+2 in Abl.** *A*, mouse Abl (amino acids 254 to C terminus) and internal deletion mutants ΔPro (lacking residues 540–645), Crkless (lacking residues 538–600), and Nckless (lacking residues 632–634) were transcribed and translated *in vitro* and binding to GST-SH3-1+2 was tested as in Fig. 1. *IVT*, input *in vitro* translation reaction before bead binding. *B*, binding of Abl and Nck *in vivo*. Full-length human Nck was expressed in 293T cells along with wt or ΔPro or Nckless mutant Abl, and Nck was precipitated with polyclonal Nck antibody (*Nck IP*) or preimmune serum (*preimm*) as control. Immunoprecipitates were subjected to immunoblotting with anti-Abl antibody to detect co-precipitating Abl. Whole cell lysates (*WCL*) were also probed with anti-Abl to confirm expression levels.

to be present by this stage of development. To confirm that Arg and Cbl were expressed at this time, we performed Northern blots of poly(A)$^+$ RNA from staged embryos probed with the *Xenopus* Arg and Cbl probes. As seen in Fig. 2, both messages were present in the unfertilized egg and throughout the cleavage and blastula stages. In the case of Arg, there was a spike of expression at stage 8 (corresponding to the mid-blastula transition, where zygotic expression begins) followed by a rapid decline after gastrulation. For Cbl, there was a large store of maternal message and relatively constant expression throughout development compared with the ornithine decarboxylase control message. We also obtained a cDNA for *Xenopus* Abl (which is highly related to Arg) and examined its expression. In this case the pattern was more complex, showing several spliced forms or related messages, but there was significant expression of all forms throughout early development. We performed whole mount *in situ* hybridization experiments to localize Arg and Cbl message. In both cases, diffuse staining was observed from the late blastula through mid-gastrula stages; this staining was strongest near the animal pole, with no obvious dorsoventral bias (data not shown). We have previously shown that endogenous Nck message is present in maternal stores and is induced at the mid-blastula transition (7). Therefore Arg, Abl, and Cbl are all expressed in the blastula and early gastrula stages, consistent with a role in the Nck-induced mesoderm patterning defect.

We next mapped the binding sites for Nck SH3 domains on Cbl and Abl. We chose to map the sites in human Cbl and murine Abl and not the *Xenopus* Cbl and Arg clones for several reasons. First, we had full-length cDNAs and a variety of mutants for the mammalian homologs available. More importantly, we wish ultimately to relate the results from *Xenopus* injection experiments to mammalian systems, where both Cbl and Abl have been shown to function as oncogenes and otherwise affect signaling. Because the *Xenopus* and mammalian versions of Cbl and Arg are so similar (66% and 64% identity for Cbl and Arg, respectively), it is reasonable to assume that their function will be conserved across species. Human Abl and Arg share over 90% identity in the region spanning the first common exon, SH3, SH2, and catalytic domains (8); although Abl and Arg differ somewhat in their biological activities (26, 27), presumably due to their fairly divergent C termini, mouse knockout experiments have shown that they are at least in part functionally redundant (9).

To map the binding sites in Abl for Nck SH3-1+2, we synthesized the catalytic domain and C terminus of c-Abl and various deletion mutants by *in vitro* transcription/translation and tested their ability to bind to GST-SH3-1+2 on beads. As shown in Fig. 3*A*, deletion of a proline-rich region of the Abl C terminus adjacent to the catalytic domain (ΔPro) eliminated binding to SH3-1+2; this region contains two binding sites for Crk SH3 domains, a nuclear localization signal, and a canonical type 2 SH3-binding site (18, 28, 29). Deletion of the two Crk binding sites ("Crkless") did not affect Nck binding, whereas deletion of the third site ("Nckless") essentially eliminated binding of Abl to SH3-1+2. These experiments confirm that the predominant binding site in Abl for Nck SH3 domains is a

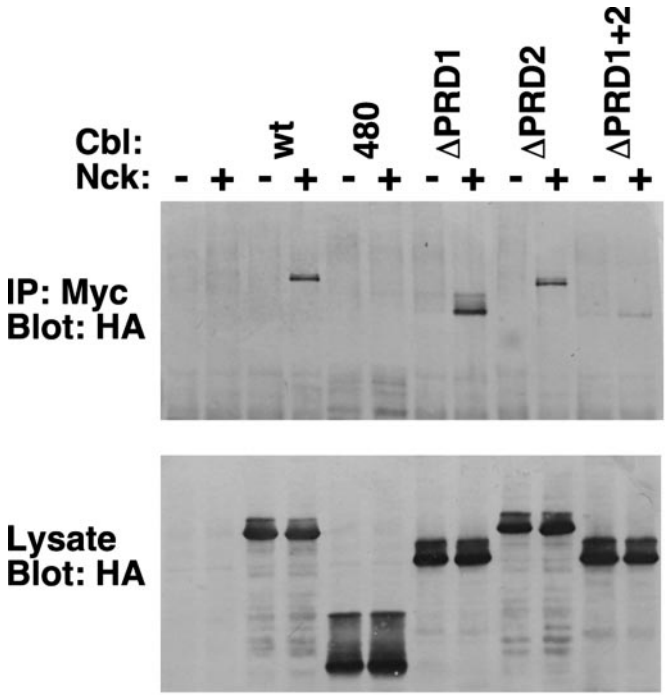

FIG. 4. **Mapping the binding site for Nck in Cbl.** Full-length human Nck (tagged with Myc epitope) and Cbl mutants (tagged with HA epitope) were cotransfected as indicated in 293T cells. Nck was immunoprecipitated with anti-Myc antibody and immunoprecipitates subjected to immunoblotting with anti-HA to detect Cbl (*top*). *Bottom panel*, whole cell lysates immunoblotted with anti-HA to detect Cbl. Mutant 480 has a deletion of aa 481–end; ΔPRD-1 lacks residues 483–688; ΔPRD-2 lacks residues 820–826; ΔPRD-1+2 has both deletions.

single P*XX*P at position 631–636, as suggested previously (18). This site is conserved between *Xenopus* Arg (APQPPKR, with canonical type 2 SH3-binding site residues underlined), human Arg, and human and mouse Abl (APTPPKR), and *Drosophila* Abl (APAPPKR).

We also examined the association of Nck with Abl *in vivo*. In lysates of 293T cells overexpressing full-length Nck and wt Abl, Abl efficiently co-immunoprecipitated with Nck (Fig. 3*B*). Mutants lacking the proline-rich Nck SH3 domain binding site also coprecipitated with Nck, albeit at somewhat lower levels. We have previously shown that binding of a Nck SH3-1+2 construct to Abl in 293T cells was almost completely eliminated by deletion of the proline-rich region of Abl (30); on the other hand, in experiments in cells expressing full-length Nck constructs, less Abl was co-precipitated with Nck SH2 domain mutants than with wt Nck.[2] Taken together, these results suggest that, in addition to the Nck SH3-mediated interaction mapped here, the SH2 domain of Nck can also contribute to Abl binding *in vivo*.

The Nck binding sites in human Cbl were identified by co-expression of full-length Myc epitope-tagged Nck with HA epitope-tagged Cbl in human 293T cells. Nck was immunoprecipitated with anti-Myc antibody, and coprecipitating Cbl was identified by immunoblotting with anti-HA antibody. As shown in Fig. 4, deletion of the entire C terminus of Cbl (mutant 480) virtually abolished Nck binding. However, deletion of the major proline-rich region of the C terminus between amino acids 483 and 688 (ΔPRD1) did not affect binding, nor did deletion of a potential type 2 SH3-binding site at amino acids 820–826 (ΔPRD2). However when these two deletions were combined in mutant ΔPRD1+2, binding to Nck was almost completely eliminated, suggesting that either of these two regions is sufficient for binding to Nck SH3 domains. Although the SH2 and SH3-3 domains are also present in wt Nck, under the conditions of this experiment, they have only a modest effect on Cbl binding.[2] The C-terminal Nck-binding site that we identified (VPERPPK) is absolutely conserved in mouse, human, and *Xenopus* Cbl, but is not present in Cbl-b or *Drosophila* Cbl; a potential variant is found in *Caenorhabditis elegans* Sli-1 (VPLPPAR).

Our major goal was to ascertain whether either Abl (or Arg) or Cbl was involved in the anterior truncation/ventralization phenotype associated with Nck overexpression in *Xenopus*. Our previous results suggested that Nck mutants exerted their ventralizing effect by recruiting *Xenopus* proteins to the membrane (7). We therefore reasoned that constitutive localization of Abl, Arg, or Cbl should ventralize in the absence of Nck mutant expression if it were the critical effector. It was impossible to test the effect of direct membrane localization of Abl and Arg, as both proteins already contain an N-terminal membrane localization signal (8, 31) and other targeting sequences (*e.g.* actin-binding and nuclear localization signals), suggesting that the protein is normally partitioned among several subcellular compartments. In the case of Cbl, we constructed a version containing an N-terminal myristoylation signal derived from Src and tested the effects of expression by injecting mRNA into the two dorsal blastomeres of four-cell stage embryos. No obvious phenotypic effects were observed (data not shown), suggesting that relocalization of Cbl to the membrane was unlikely to be uniquely responsible for the Nck-induced phenotype.

We also reasoned that if binding of Nck to endogenous Abl, Arg, or Cbl was responsible for the phenotype, overexpression of these proteins should enhance the ventralizing effect of Nck. The Lys-229 mutant of Nck, in which SH3-3 is mutated but all other domains are intact, is the most potent Nck mutant for anterior truncation/mesoderm ventralization (7). When Nck Lys-229 is expressed at low levels (100 pg of RNA/embryo), however, the great majority of embryos develop normally (Table I and Fig. 5). This sensitized system allowed us to test the effects of overexpression of candidate Nck effectors. As shown in Table I and Fig. 5, overexpression of Abl or Cbl alone did not enhance anterior truncation by Nck Lys-229. However, the combination of Nck Lys-229, Abl, and Cbl induced a phenotype that was much stronger than that induced by injection of any of the RNAs individually or pairwise. The effect of wt Nck, which we had previously shown to be less potent than the Lys-229 mutant (7), was also enhanced by the combination of Abl and Cbl (Table I). We eliminated the possibility that this phenotype was simply due to increased levels of injected RNA, as neither co-injection of Abl and Cbl with a Nck mutant in which all binding domains are mutated (Nck Kall), nor co-injection of Nck Lys-229 and Abl with a Cbl mutant that cannot bind Nck (Cbl Δ1+2), could induce anterior truncation (Table I). Finally, human Arg behaved identically to Abl in these assays, synergizing with Cbl and Nck Lys-229 (Table I). Together, these results imply that anterior truncation/ventralization induced by Nck involves both Cbl and Abl family kinases.

We also examined the effects of overexpressing wild-type c-Abl and an activated, transforming version of Abl alone. We have previously shown that expression of Nck SH3 domains can activate the normally repressed c-Abl protein in human 293T cells (30). We considered the possibility that, in *Xenopus* embryos, one consequence of Nck overexpression might be the activation of c-Abl, leading in turn to ventralization and anterior truncation. Various amounts of RNA encoding wt c-Abl or an SH3-deleted, constitutively active form were injected and ventralization was assessed by dorsoanterior index as above.

[2] T. Miyoshi-Akiyama, L. M. Aleman, J. M. Smith, C. E. Adler, and B. J. Mayer, submitted for publication.

TABLE I
Injected mRNAs

mRNAs indicated were injected into the two dorsal blastomeres of four-cell-stage embryos and DAI was scored at stage 35. DAI 5 embryos are normal, whereas smaller numbers indicate increasing dorsoanterior deficiencies (22). Representative data from several experiments are presented; all experiments were repeated several times with essentially similar results.

mRNA injected	pg RNA/embryo	DAI			No. injected (% survival)
		5	4–3	2–1	
Experiment 1					
Lys-229	100	96.6	3.4	0	140 (96.7)
Cbl	100	100	0	0	50 (96.0)
Abl	50	89.1	10.9	0	75 (73.3)
Lys-229 + Abl	100 + 50	75	25	0	50 (64.0)
Abl + Cbl	50 + 100	80.9	19.1	0	75 (90.7)
Lys-229 + Cbl	100 + 100	100	0	0	75 (97.3)
Lys-229 + Abl + Cbl	100 + 50 + 100	45.7	42.9	11.4	50 (96.0)
Experiment 2					
Lys-229 + Abl + Cbl	100 + 50 + 100	24.3	71.4	4.3	75 (93.3)
wt Nck + Abl + Cbl	100 + 50 + 100	47	50	3	75 (88.0)
Nck Kall + Abl + Cbl	100 + 50 + 100	80.6	17.9	1.5	75 (89.3)
Lys-229 + Abl + CblΔ(1 + 2)	100 + 50 + 100	82	18	0	75 (97.3)
Experiment 3					
Arg	50	93.3	5.3	1.3	75 (100)
Arg + Cbl	50 + 100	97.3	2.7	0	75 (90.1)
Lys-229 + Arg	50 + 100	76.7	20.1	2.9	75 (100)
Lys-229 + Arg + Cbl	50 + 100 + 100	49.2	42.3	8.6	75 (94.7)

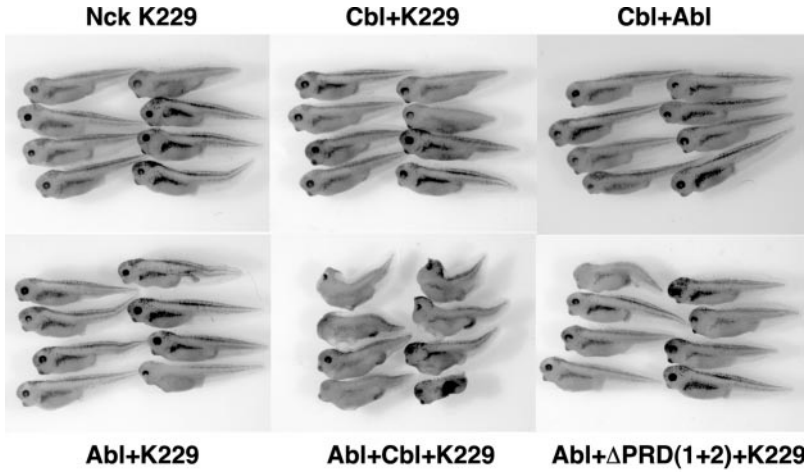

FIG. 5. **Combination of Nck, Cbl, and Abl induces anterior truncation/ventralization phenotype.** 100 pg/embryo mRNA encoding Nck Lsy-229, with or without 100 pg/embryo wt Cbl or CblΔPRD-1+2 (which cannot bind Nck), and/or 50 pg/embryo wt c-Abl, were injected into the two dorsal blastomeres of four-cell-stage embryos in combinations indicated. Photomicrographs of representative embryos were taken at stage 35 (28–36 h after fertilization).

As shown in Fig. 6, activated Abl very potently induced anterior truncation in a dose-dependent manner, with 50% of embryos affected at a dose of 25 pg of RNA. By comparison, although unmutated c-Abl also induced anterior truncation, it did so only at much higher levels of expression (50% of embryos affected at a dose of 500 pg of RNA/embryo). Expression of wt human Arg also induced anterior truncation with a dose dependence similar to that of wt c-Abl. Injection of RNA encoding wt Cbl or the myristoylated version at amounts up to 2 ng/embryo did not have any obvious effect, either alone or in combination with wt or activated Abl (data not shown).

DISCUSSION

In this study we have identified the Cbl proto-oncogene product and the Abl and Arg nonreceptor tyrosine kinases as likely effectors of Nck-induced anterior truncation in *Xenopus*. Protein binding modules such as SH3 domains are inherently only quasispecific, as their interactions with many proteins have similar affinities (32). Therefore, in order to determine whether a particular SH3-binding protein plays a role in a certain process, we must establish criteria to validate the interaction. In the case of Nck-induced ventralization, a number of such criteria were available, allowing us to assess the relevance of various Nck-binding proteins to the phenotype. First, we asked whether a candidate bound preferentially to a construct containing both SH3-1 and SH3-2, since our previous results suggested that both domains were required in cis for ventralization. Out of 39 SH3-1+2 binding clones isolated from an expression library, only *Xenopus* homologs of Cbl and Arg fulfilled this first criterion. Second, we showed that transcripts for Cbl and Arg (and the Arg-related protein Abl) were expressed in *Xenopus* embryos during the blastula and early gastrula stages, when mesoderm induction and patterning are known to occur (reviewed in Refs. 33 and 34). Third and most significantly, overexpression of the combination of Abl (or Arg) and Cbl potentiated the activity of subthreshold amounts of ventralizing Nck mutants. Taken together, these results implicate both Cbl and Abl family kinases in the aberrant pattern formation induced by Nck overexpression, and suggest that these proteins might function together in normal mesoderm patterning.

Interestingly, the two proteins that emerged from the screen share striking similarities in biological activity. Both Cbl and Abl were originally isolated as retroviral oncogenes, indicating that mutated versions can induce the unregulated proliferation that is the hallmark of cancer. On the other hand, for both Cbl and Abl, the unmutated proto-oncogene product is associated with repression of proliferation and/or signaling: Abl overexpression is cytostatic, inducing G_1 arrest (reviewed in Refs. 10 and 11), whereas loss of function of the *C. elegans* Cbl homolog

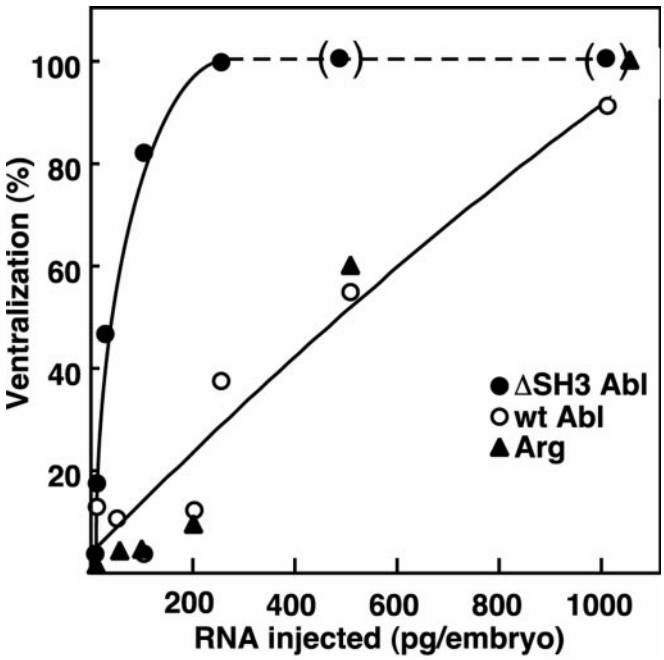

FIG. 6. **Ventralization induced by Abl and Arg.** Various amounts of mRNA encoding wt mouse c-Abl, wt human Arg, and an activated, SH3-deleted version of Abl (ΔSH3 Abl) were injected in the two dorsal blastomeres of four-cell embryos. At least 25 embryos were injected, and the DAI was scored at stage 36. Percentage of ventralized embryos (scored as DAI 0–4) are indicated. Symbols in *parentheses* mean that the survival rates were less than 80% in this experiment.

Sli-1 restores normal levels of signaling to a compromised receptor tyrosine kinase (12). Therefore, both Cbl and Abl (and its close relative Arg) are excellent candidates to modulate mesoderm patterning, which is governed by the ability of cells to interpret their position in gradients of extracellular signals and respond to that positional information appropriately (33, 34).

Our results imply that both Cbl and Abl (or Arg) are coordinately involved in the ventralization/anterior truncation induced by Nck, because the combination of all three proteins strongly potentiated anterior truncation. Of course, at higher levels of expression, we have previously shown that Nck alone is sufficient to ventralize embryos (7), and we further show here that Abl alone can also ventralize when highly overexpressed. Therefore, the simplest interpretation of our data is that Cbl overexpression increases the signaling output induced by Nck and Abl. Our previous results had suggested that, in presumptive mesoderm cells, Nck can assemble complexes at sites of tyrosine phosphorylation on membranes; recruitment of proteins binding to the first two SH3 domains then induces cells to adopt a ventral fate (7). By overexpressing Abl and Cbl, we are presumably increasing the amount of Nck-Abl and Nck-Cbl complexes at sites of tyrosine phosphorylation on the membrane. We have recently shown that relocalization of Abl via Nck SH3 domains can activate its tyrosine kinase activity in mammalian cells (30), so one possible consequence of overexpression of Nck and Abl in *Xenopus* embryos is the activation of Abl. We show here that overexpression of low levels of activated mutant Abl or high levels of wt c-Abl alone is in fact sufficient to induce anterior truncation in these assays (Fig. 6), consistent with a role for activated Abl in Nck-induced phenotypes.

The role of Cbl in potentiating the activity of low amounts of Nck and Abl is particularly intriguing. Because Cbl can mediate the ubiquitination and destruction of associated proteins (13–16), it might be expected that recruitment of Cbl via Nck to sites of tyrosine phosphorylation would lead to their downregulation, an activity seemingly in opposition to the kinase activity of Abl (which has been suggested to play a positive role downstream of tyrosine kinases such as the PDGF receptor (Ref. 35)). One possible interpretation is that phosphorylation of Cbl itself is critical for ventralization; this phosphorylation would be facilitated by Nck-mediated relocalization and by Abl activity. We have shown that Cbl is very efficiently phosphorylated by Abl in 293T cells.[2] Phosphorylation of Cbl is likely to have a number of effects, from recruiting other binding partners such as the Crk adaptor and phosphatidylinositol 3-kinase (reviewed in Ref. 36) to stimulating its ubiquitin ligase activity (16). Therefore, it is reasonable to assume that simultaneous overexpression of Nck, Cbl, and Abl might lead to partial activation of Abl and elevated levels of phosphorylated, biologically active Cbl at specific sites in the cell.

What are the upstream signaling pathways being affected? One candidate is the FGF1 receptor, which has been shown to be involved both in the induction and patterning of mesoderm (Ref. 37; reviewed in Refs. 38 and 39) and has also been shown to bind Nck (40). Hyperstimulation of the FGF pathway would be consistent with the activity of Nck overexpression in whole embryos. However, we previously showed that wt and Lys-229 mutant Nck, both of which can induce anterior truncation in whole embryos, also block FGF-mediated activation of mitogen-activated protein kinases in animal cap explants, suggesting an inhibitory and not stimulatory role for overexpressed Nck (41). A second possible upstream factor is the PDGF receptor, which is required for fibronectin-mediated adhesion of mesodermal cells during gastrulation (42, 43). Inhibition of the PDGF receptor would prevent appropriate migration of the dorsal mesodermal cells to the anterior, consistent with the Nck anterior truncation phenotype. In contrast with Nck mutant overexpression, however, PDGF receptor inhibition is not associated with ventralization of mesoderm in animal caps (7, 42). In this regard it should be noted that, in our earlier experiments, high levels of expression of strongly ventralizing Nck mutants were required to induce detectable ventralization in animal caps, whereas much lower levels were required to induce anterior truncation in whole embryos (7). Furthermore, we have not observed induction of ventral markers in activin-induced animal caps from embryos injected with low levels of Nck, Abl, and Cbl (data not shown), suggesting that dorsoventral fate need not be directly affected by Nck in all cases.

Regardless of the specific upstream signals affected by Nck overexpression, our results provide strong evidence that Abl family kinases and Cbl play an important role in the patterning of the early *Xenopus* embryo. The fact that overexpression of both Cbl and Abl is required to enhance the Nck-induced phenotype suggests they work together in the same pathway, perhaps as a multiprotein complex. All three proteins can be shown to interact with each other, not only by SH3-mediated interactions as shown here but also by SH2-phosphotyrosine interactions.[2] Given the functional data presented here suggesting a common role in signaling, it will be of great interest to dissect the biochemical and physiological consequences of their interaction.

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MOLECULAR BASIS OF CELL AND DEVELOPMENTAL BIOLOGY:

Abl Family Kinases and Cbl Cooperate with the Nck Adaptor to Modulate *Xenopus* Development

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