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An Evolutionarily Conserved Transmembrane Protein That Is a Novel Downstream Target of Neurotrophin and Ephrin Receptors

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Appropriate development of nervous system connectivity involves a variety of processes, including neuronal life-and-death decisions, differentiation, axon guidance and migration, and synaptogenesis. Although these activities likely require specialized signaling events, few substrates unique to these neurotrophic functions have been identified. Here we describe the cloning of ankyrin repeat-rich membrane spanning (ARMS), which encodes a novel downstream target of neurotrophin and ephrin receptor tyrosine kinases, Trk and Eph, respectively. The amino acid sequence of ARMS is highly conserved from nematode to human, suggesting an evolutionarily conserved role for this protein. The ARMS protein consists of 1715 amino acids containing four putative transmembrane domains, multiple ankyrin repeats, a sterile α motif domain, and a potential PDZ-binding motif. In the rat, ARMS is specifically expressed in the developing nervous system and in highly plastic areas of the adult brain, regions enriched in Trks and Eph receptors. ARMS can physically associate with TrkA and p75 neurotrophin receptors. Moreover, endogenous ARMS protein is tyrosine phosphorylated after neurotrophin treatment of pheochromocytoma 12 cells and primary hippocampal neurons or ephrin B treatment of NG108-15 cells, demonstrating that ARMS is a downstream target for both neurotrophin and ephrin receptors.

Key words: neurotrophin; Trk; p75; ephrin; Eph; tyrosine kinase; tyrosine phosphorylation; ankyrin

The formation of the nervous system requires appropriate connectivity of neurons and their targets both spatially and temporally. Two families of proteins that mediate these actions are the neurotrophins and ephrins. Neurotrophins play a prominent role in the development of the vertebrate nervous system by influencing cell survival, differentiation, and cell death events (Levi-Montalcini, 1987; Lewin and Barde, 1996). Neurotrophins also exhibit acute regulatory effects on neurotransmitter release, synaptic strength, and connectivity (Thoenen, 1995; Bonhoeffer, 1996; McAllister et al., 1999). In addition to promoting axonal (Patel et al., 2000) and dendritic branching, neurotrophins serve as chemoattractants for extending growth cones *in vitro* (Letourneau, 1978; Gundersen and Barrett, 1979; Gallo et al., 1997).

Ephrins comprise another class of ligands that function in axon guidance, cell migration, axon fasciculation, boundary formation, topographic mapping, and morphogenesis (Frisén et al., 1999). Ephrins are a family of eight proteins that are found associated with the plasma membrane, either via a glycosylphosphatidylinositol linkage (as seen in the A subfamily) or as transmembrane proteins (as seen in the B subfamily). Ephrins signal via receptor protein tyrosine kinases, but the biological outcomes are distinct from mitogenic factors such as platelet-derived growth factor and epidermal growth factor (EGF), both of which transmit signals via tyrosine phosphorylation (Brückner and Klein, 1998; Flanagan and Vanderhaeghen, 1998; Holland et al., 1998). Ephrin receptor-associated molecules such as Crk, Nck, RasGAP, and Fyn are proposed links between the receptor and downstream events such as cell adhesion and cytoskeletal changes. In addition, Grb2, Grb10, and the p85 subunit of phosphatidylinositol-3 kinase (PI-3 kinase) are used in ephrin receptor signal transduction (Mellitzer et al., 2000).

The neurotrophins [NGF, BDNF, neurotrophin-3 (NT-3), and NT-4/5] exert their effects via two classes of receptors (Kaplan and Miller, 2000). TrkA, TrkB, and TrkC serve as receptor tyrosine kinases for NGF, BDNF and NT-4, and NT-3, respectively (Chao, 1992a). The p75 receptor is a member of the tumor necrosis factor receptor superfamily (Smith et al., 1994) and binds to all neurotrophins. Most central and peripheral neurons express p75 together with one or more of the Trks. The p75 receptor, when coexpressed with TrkA, provides a positive influence on Trk function (Barker and Shooter, 1994; Verdi et al., 1994) and determines the specificity of neurotrophin binding and responsiveness (Benedetti et al., 1994; Bibel et al., 1999; Brennan et al., 1999). An association of p75 and Trk receptors has been detected by coprecipitation (Huber and Chao, 1995; Gargano et al., 1997; Bibel et al., 1999), and copatching of these receptors has been observed by the use of fluorescent-labeled antibodies (Ross et al., 1996).

Receptor tyrosine kinases frequently use a number of common intracellular signaling components such as phospholipase C-γ, PI-3 kinase, and adaptor proteins such as Shc and Grb2. Common to many of these proteins is their ability to bind to phosphorylated tyrosines via domains such as the Src homology-2 (SH2) and

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phosphotyrosine-binding (PTB) domains. How these shared signaling components lead to different biological outcomes is not well understood (Chao, 1992b). Possible mechanisms include receptor use of substrates in a differential manner (e.g., differential association/dissociation kinetics), competition for binding between different substrates (Meakin et al., 1999), or recruitment of unique target proteins, such as rAPS and SH2-B (Qian et al., 1998).

To define proteins associated with the neurotrophin receptors, we used the intracellular domain of the p75 neurotrophin receptor as bait in a two-hybrid screen of a dorsal root ganglion library. Here we report the properties of a novel transmembrane protein, designated ARMS (ankyrin, repeat rich, and membrane spanning), that contains a number of interesting features, including multiple ankyrin repeats, four putative transmembrane domains, a sterile α motif (SAM) domain, and a consensus PSD95/SAP90, DLG, and ZO1 (PDZ)-binding motif. Interestingly, ARMS does not contain SH2 or PTB domains, suggesting that it confers signaling specificity downstream of receptor tyrosine kinases in a manner distinct from classical adaptor proteins. An analysis of the structure and distribution of this protein suggests that it may function during development of the nervous system. Most importantly, ARMS is phosphorylated after treatment with NGF, BDNF, and ephrin B2, suggesting that it is a critical link between cell surface receptors and intracellular signaling events for both the neurotrophin and the ephrin families.

MATERIALS AND METHODS

Materials. NGF was obtained from Harlan (Indianapolis, IN), BDNF was from Peprotech (Rocky Hill, NJ), NT-4/5 was a generous gift of Regeneron (Tarrytown, NY), EGF was obtained from Intergen (Purchase, NY), and K252a was from Calbiochem (La Jolla, CA).

Construction of the two-hybrid DRG library and yeast two-hybrid screen. A cDNA library was constructed into unique *Bst*XI–*Not*I sites of a modified version of pJG4-5. Briefly, polyadenylated RNA, which had been purified from adult mouse and postnatal day 1 (P1) rat DRG by the use of Trizol (Life Technologies, Gaithersburg, MD) and the PolyA Tract System (Promega, Madison, WI), was used as the template for reverse transcription (Life Technologies) with an oligo-dT–*Not*I primer. Subsequent ligation into pJG4-5 and electroporation into DH5α yielded a library of $\sim 10^6$ cfu and an average insert size of 1.5–2.0 kb. A two-hybrid interaction screen, based on the LexA system (Gyuris et al., 1993), was performed in EGY48. The bait consisted of the entire cytoplasmic region of rat p75 as an in-frame fusion with the LexA DNA-binding domain. Library cDNAs were expressed as in-frame fusions with the Gal4 transcriptional activation domain. Approximately 90 million yeast transformants were screened for their ability to survive in the absence of leucine. Sequence analysis identified a novel cDNA clone of ~2.5 kb that corresponded to the C-terminal 250 amino acids of ARMS.

Isolation of ARMS cDNA. Several rat brain libraries (young adult whole brain, adult whole brain, and adult hippocampus) were screened to obtain the full-length ARMS cDNA. Criteria used to establish the initiator methionine include the following: assessment of multiple, independent cDNA fragments spanning the start site, an upstream, in-frame stop codon, and conformity to the Kozak consensus sequence. The ARMS sequence has been deposited into the GenBank database under accession number AF313464.

Northern blotting and in situ *hybridization.* Total RNA was extracted from various rat tissues using Trizol reagent (Life Technologies). Twenty micrograms of total RNA were loaded per lane (with the exception of pancreas and DRG RNAs, which were <10 μg/lane), electrophoresed through a 2.2 M formaldehyde and 1% agarose gel, transferred to a nylon membrane (Qiagen, Santa Clarita, CA), baked for 2 hr at 80°C, and probed with a cDNA fragment of ARMS labeled with [^{32}P]dCTP using Ready-To-Go (Amersham Pharmacia Biotech, Piscataway, NJ). *In situ* hybridization was performed as described previously (Lai and Lemke, 1991). Briefly, 30 μm paraformaldehyde-fixed brain sections from adult rat or whole embryos were slide-mounted, hybridized with a ^{33}P-labeled cRNA probe generated by T7 RNA polymerase from a PCR-generated ARMS fragment, and washed at a final stringency of 0.1× SSC at 60°C for 35 min. Emulsion-dipped slides were exposed for various times (days to weeks) before developing and then counterstained with thionin.

Cell culture. Human embryonic kidney (HEK) 293 and 293T cells were maintained in DMEM containing 10% fetal bovine serum (FBS) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Native pheochromocytoma (PC) 12 cells, TrkA-overexpressing PC12 cells (615) (Hempstead et al., 1992), and TrkB-overexpressing PC12 cells were maintained in DMEM containing 10% FBS and 5% heat-inactivated horse serum with 30 U/ml penicillin, 30 μg/ml streptomycin, 2 mM glutamine, and 200 μg/ml G418.

SCG neurons were prepared from P2 rats and cultured on collagen-coated coverslips in C-medium (minimum essential medium containing 10% FBS supplemented with 0.4% glucose and 2 mM L-glutamine) with 150 ng/ml 2.5 S NGF (Harlan). To inhibit growth of non-neuronal cells, neurons were cultured in the presence of 24.6 mg/ml 5-fluoro-2-deoxyuridine and 24.4 mg/ml uridine.

Dissociated primary cultures of hippocampal neurons from embryonic day 17 (E17) rats were prepared from timed-pregnant Sprague Dawley rats following published procedures (Aibel et al., 1998). After dissection of the hippocampus, the meninges were removed. The tissue was briefly minced with fine forceps and then triturated through a fire-polished Pasteur pipet. Cells were counted and plated in Neurobasal media supplemented with B27 (Life Technologies) on cell culture dishes coated overnight with 0.01 mg/ml poly-D-lysine. Cells were grown in a humidified incubator with 5% CO_2 at 37°C.

Antibodies. To characterize the expression of the ARMS protein, we generated a polyclonal antibody (892) against the C terminus of ARMS. A bacterially expressed GST fusion protein with the C-terminal 180 amino acids of ARMS was purified and used as an antigen to generate rabbit antiserum (Cocalico Biologicals, Reamstown, PA). The specificity of this antiserum was determined by Western blot analyses of HEK293T cells transfected with a FLAG-tagged, full-length cDNA of ARMS. Lysates of transfected HEK293T cells, but not untransfected cells, displayed a specific 190 kDa species after immunoblotting with either an anti-FLAG antibody or anti-ARMS serum 892.

Transfection of mammalian cells, immunoprecipitation, and immunoblotting. For transient transfection experiments, HEK293 or HEK293T cells plated at 70–80% confluency in 10 cm dishes were subjected to calcium phosphate transfection with different combinations of the mammalian expression plasmids containing cDNAs for ARMS, rat TrkA, and rat hemagglutinin (HA)-tagged p75 (Khursigara et al., 1999). PC12 615 cells or transiently transfected cells were harvested and lysed in 1 ml of 10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 1% NP-40 (TNE buffer) containing 0.12 mg/ml phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 1 μg/ml aprotinin, 10 mM NaF, and 1 mM Na_3VO_4 for 30 min on ice. After centrifugation and removal of the insoluble fraction, the protein concentration of the supernatant was determined by the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. Cell lysates of equivalent protein content were incubated for 4 hr to overnight with rotation at 4°C with either anti-pan-Trk polyclonal antibody, C14 (1.5 μg), or anti-ARMS 892 antiserum (1:100). The immune complexes were immobilized on protein A-Sepharose beads (Sigma, St. Louis, MO), washed six times with ice-cold TNE buffer, boiled in SDS-sample buffer, separated by SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Immunoblotting was performed by first blocking membranes in 20 mM Tris, pH 7.5, 500 mM NaCl, and 0.1% Tween 20 (TBST buffer) containing 5% BSA for pY99 and 5% nonfat milk for others and then incubating for 2 hr at room temperature or overnight at 4°C in TBST buffer containing one of the following primary antibodies: anti-Trk 44 serum (1:2000); anti-phosphotyrosine pY99 antibody (0.1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA); or anti-ARMS 892 antiserum (1:1000). Membranes were washed with TBST buffer and then incubated with horseradish peroxidase-conjugated goat anti-rabbit (Roche Molecular Biochemicals, Indianapolis, IN) or goat anti-mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:10,000 or 1:7500, respectively. Immunoreactive protein bands were detected by enhanced chemiluminescence using ECL reagents (Amersham Pharmacia Biotech).

Immunofluorescence analysis. SCG neurons were fixed with 4% paraformaldehyde and blocked with PBS containing 0.075% saponin and 10% FBS or normal goat serum. Cells were then incubated with anti-ARMS 892 (1:1000) or anti-Trk B-3 antibody (1 μg/ml) in blocking buffer. Primary antibodies were visualized using fluorescence-conjugated sec-

ondary antibodies [indocarbocyanine-conjugated goat anti-mouse IgG (Jackson ImmunoResearch; 1:200) or FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch; 1:100)]. Images were collected on a Bio-Rad confocal microscope.

RESULTS

Cloning of ARMS

Neurotrophins exert many biological activities, but few signaling molecules have been found to be specific for neurotrophin receptor signaling. The majority of proteins that serve as substrates for neurotrophin receptors are used by other receptor families. Effects on cellular and axonal migration, nerve regeneration, and apoptosis have been implicated for the p75 neurotrophin receptor, and effects on long-term potentiation and synaptic transmission have been attributed to the Trks. To define unique molecules in neurotrophin signaling, a two-hybrid screen was undertaken using a rat dorsal root ganglion library as prey and the cytoplasmic domain of p75 as bait. This report describes a p75-interacting protein that is a downstream target for Trk signaling.

A positive cDNA clone was identified that encoded the C-terminal portion of a novel 1715 amino acid protein. The predicted amino acid sequence contained 11 contiguous, 33 amino acid ankyrin repeats in the N-terminal domain and four putative membrane-spanning regions. We have designated this protein ARMS. The overall topology of ARMS is decidedly different from that of other transmembrane proteins. Other distinguishing features of this protein include a SAM domain, a polyproline stretch, and a potential PDZ-binding motif (Fig. 1*A,B*). These motifs represent candidate protein–protein interaction domains.

An extensive search of GenBank databases for proteins similar to ARMS identified many ankyrin-containing proteins; however, proteins sharing homology with ARMS in regions outside of the ankyrin repeats were notably absent. Analysis of *Caenorhabditis elegans*, *Drosophila*, and human databases revealed ARMS orthologs in these organisms. The presence and conservation of ARMS sequences from nematodes to humans suggest that this protein may serve evolutionarily conserved functions. Between rat and human, the amino acid identity is 91%, and similarity is 94% (Fig. 1*B*). Comparison of ARMS sequences among human, rat, *Drosophila*, and *C. elegans* revealed similarity in overall structure: multiple ankyrin repeats in the N terminus, four putative transmembrane domains, SAM domains, and consensus PDZ-binding motifs at the three carboxymost amino acids (SIL in human and rat, TKL in *Drosophila*, and SDA in *C. elegans*). Despite the relatively divergent C termini among the ARMS homologs, the presence of potential PDZ recognition sequences in all of these proteins indicates that conserved protein–protein interactions for ARMS may exist.

Interestingly, the regions most highly conserved were not the transmembrane (TM) domains but certain regions surrounding them, namely, the ankyrin repeats, the N-terminal portion between the ankyrin repeats and the first TM domain, the "loop" region between TM domains 2 and 3, and several stretches of amino acids in the C terminal downstream of TM4, including the SAM domain (Fig. 1*C*). The regions shown in Figure 1*C* range between 20 and 42% identical and 56 and 77% similar, whereas the TM domains are only 1% identical and 43% similar. It is interesting to note that embedded in these subdomains are conserved tyrosine residues; these potential phosphorylation sites are conserved among all four organisms examined (Fig. 1*C*, *part 1*).

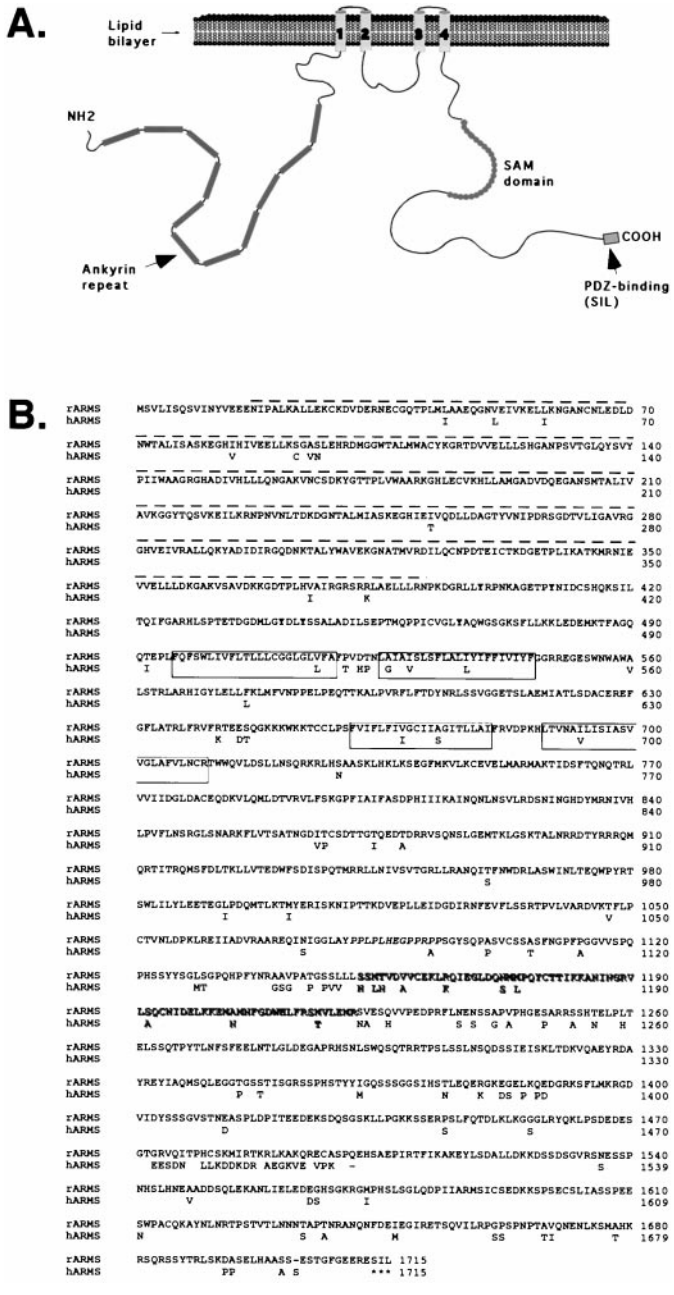

Figure 1. ARMS represents a novel transmembrane protein. *A*, Predicted topology of ARMS. Transmembrane domains (labeled 1–4) and various intracellular motifs are depicted. *B*, Amino acid sequence and comparison of rat ARMS (rARMS) and human ARMS (hARMS) proteins. Residues marked with a *dashed line* denote 11 contiguous ankyrin repeats; *bold-faced* tyrosine (Y) residues (at positions 399, 409, 441, 444, and 466 of the rat sequence) are evolutionarily conserved among human, rat, *Drosophila*, and *C. elegans*; *boxed* residues are the putative transmembrane domains; *italicized* residues denote the polyproline stretch; *shadowed* residues constitute the SAM domain (amino acids 1152–1221); and the three carboxymost amino acids marked with *asterisks* (SIL) encode a potential PDZ-binding motif. Amino acids of the human sequence that differ from the rat sequence are shown. (*Figure 1 continues*.)

Although mutations in *C. elegans* and *Drosophila* ARMS have not yet been described, the *C. elegans* homolog F36H1.2 is most similar to the ankyrin-related gene UNC-44 (28%), which has a role in axon guidance, axonogenesis, and neuronal development (Otsuka et al., 1995).

C.

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1.  Amino terminus
rARMS       354 LLLDKGAKVSAVDKKGDTPLHVAIRGRSRRLAELLLRNPKDGRLLYRPNKAGETPYNIDCSHQKSILTQI
hARMS       354 LLLDKGAKVSAVDKKGDTPLHIAIRGRSRKLAELLLRNPKDGRLLYRPNKAGETPYNIDCSHQKSILTQI
dARMS       376 LLLDRKAKVTASDKRGDTCLHIAMRARSKTIVEALLRNPKHSQLLYRANKAGETPYNIDSLHQKTILGQV
wARMS       343 YLMSFGAKLAAVDNCGDNALHLALRARSRRLTQALLSNPSDSRLLYRPNKLGQTPYSIDLSNPQPILPLI
                 *:.  **::* *: **. **:*:*.**: :.: ** **...:**.** *:**.**  : :.**  :

rARMS           FGARHLSPTETDGDMLGYDLYSSALADILSEPTMQPPICVGLYAQWGSGKSFLLKKLEDEMKTFAGQQTE 493
hARMS           FGARHLSPTETDGDMLGYDLYSSALADILSEPTMQPPICVGLYAQWGSGKSFLLKKLEDEMKTFAGQQIE 493
dARMS           FGARRLNTNEDSEGMLGYELYSSALADVLSEPTLTTPITVGLYAKWGSGKSFLLNKLRDEMNNFARQWAE 515
wARMS           FGP--IDAEDKMDTAMGYDVYSNVLADIVCEPSLSLPLTIGLYAKWGSGKSALLAKLKEAMHSFSRDWLD 480
                **.  :.. :     :**.::**..***::.**::   *: :***.*:****** ** **.: *:.*: :  :

2.  Loop between TM2 and TM3
rARMS       573 ELLFKLMFVNPPELPEQTTKALPVRFLFTDYNRLSSVGGETSLAEMIATLSDACEREFGFLATRLF 638
hARMS       573 ELLLKLMFVNPPELPEQTTKALPVRFLFTDYNRLSSVGGETSLAEMIATLSDACEREFGFLATRLF 638
dARMS       594 RLILQVAFCHPP-GPQSDSQAKPVRFHFAEANSASPTG-DGAVAHMLAALLDAIESHYGWLATRLY 657
wARMS       565 RLVYNILTLHAP-MNSEDSASMPVSFLFADYHRLSSIGGEQALAKIVATLFEAAETHFGVLPVRLF 629
                .*: ::   :.*   .. : : ** * *:: :  *. * : ::*.::*:* :* * .:* *..**:

3.  Carboxy tail
rARMS       749 EVELMARMAKTIDSFTQNQTRLVVIIDGLDACEQDKVLQMLDTVRVLFSK---GPFIAIFASDPHIIIKA
hARMS       749 EVELMARMAKTIDSFTQNQTRLVVIIDGLDACEQDKVLQMLDTVRVLFSK---GPFIAIFASDPHIIIKA
dARMS       740 EVAVMTDMVKCLDAFTNQQSRLVGVIDALDSCDTERILTLLNAVQTLLSS-PNRPFVLLISVDPHVIAKA
wARMS       769 EVDLLADMIRSLDAFTRSHTRLVVVVDGLDNCEQERMVQTLDALELLFSARKHRPFITIIAVDPHVIVSA
                ** ::: * : :*:**.:::*** ::*.** *: ::::  *:::. *:*      **: ::: ***:* .*

rARMS           INQNLNSVLRDSNINGHDYMRNIVHLPVFLNSRGLSNAR 854
hARMS           INQNLNSVLRDSNINGHDYMRNIVHLPVFLNSRGLSNAR 854
dARMS           AEANSRRLFTEGGIGGHDFLRNLVHLPVYLQNSGLRKVQ 848
wARMS           INHNMHSALSGTELTGHDYLKNIISMPFYLHNSALRQLQ 876
                 : * .  :    : ***:::*:: :*.::*:. .* : :

4.  Carboxy tail
rARMS       940 MRRLLNIVSVTGRLLRANQITFNWDRLASWINLTEQWPYRTSWLILYLEETEG--LPDQMTLKTMYERIS
hARMS       940 MRRLLNIVSVTGRLLRANQISFNWDRLASWINLTEQWPYRTSWLILYLEETEG--IPDQMTLKTIYERIS
dARMS       980 MRRLMNVIYITVRLLKAFQIEFSWYRLSSWINLTEQWPLRASMIVLHHDQFMDSNADESVSLQSVYEKLR
wARMS       923 MRRIVNALTLTGRLMRAFEIDFSWMSLGHWVSLLEQWPSRMCWLIDRALEVHN----NQLLLSEVYYQLK
                ***::* : :* **::* :* *.*  *. *:.* **** * . ::    :  .    ::: *. :* ::

rARMS           KNIPTTKDVEPLLEIDGDIRNFEVFLSSR----TPVLVARDVKTFLPCTVNLDPKLR 1060
hARMS           KNIPTTKDVEPLLEIDGDIRNFEVFLSSR----TPVLVARDVKVFLPCTVNLDPKLR 1060
dARMS           PKLAYLREAAPLLELDRDERKLDAFLQLH----KSDLLVADLRIFLPFTINLDPYLR 1102
wARMS           DHIP---AQDDLMQLDRNLENFEGFLDSKGIPSAERLTVGHVKKFVPCTSSLDPYLR 1042
                 ::.       *:::* : .::: **. :       * . .:: *:* * .*** **

5.  SAM domain
rARMS       1151 LSSMTVDVVCEKLRQIEGLDQNMMPQYCTTIKKANINGRVLSQCNIDELKKEMAMNFGDWHLFR 1214
hARMS       1151 LNSLNVDAVCEKLKQIEGLDQSMLPQYCTTIKKANINGRVLAQCNIDELKKEMNMNFGDWHLFR 1214
dARMS       1193 LTDLTVEGVISLLDRIEDMKP-ALPKLAPVLRENAINGRVLKHCDMPDLKSVLGLSFGHWELFR 1255
wARMS       1087 LVEMKLDAVVNLIRKIDIPSN-RLDSILDRFYQLNLCGLVLATCPLPELKDSMQLPLGDWTLIR 1149
                 * .:.:: * . : :*:  .   : .    : :  : * **  * : :**. : : :*.* *:*
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(*Figure 1 continued*.) *C*, Comparison of various cytoplasmic regions of rat (*r*), human (*h*), *Drosophila* (*d*), and *C. elegans* (*w*) ARMS proteins. *Part 1*, N-terminal region between the ankyrin repeats and the first transmembrane domain with *bold-faced* evolutionarily conserved tyrosines (Y). *Part 2*, Cytoplasmic region between transmembrane domains 2 and 3. *Parts 3, 4*, Two C-terminal regions. *Part 5*, The SAM domain. Sequences for wARMS, dARMS, and hARMS were obtained from accession numbers Z68760, AAF46710, and BAA86564/CAB63746, respectively. *Symbols*: *asterisk*, identity; *colon*, strongly similar; *period*, weakly similar.

Distribution of ARMS transcripts in rat

In rat tissue, expression of ARMS mRNA was assessed by Northern blot analysis using a probe directed against the membrane-spanning regions of ARMS (Fig. 2, *top*). A single transcript of ~7.0 kb was detected. Although ARMS mRNA could be detected in several non-neuronal tissues, it was most abundant in the nervous system.

We examined the expression of ARMS by *in situ* hybridization in adult rat brain. Several populations of neurons were found to express ARMS mRNA, including mitral cells and cells of the glomerular layer of the olfactory bulb, all regions of the hippocampus, Purkinje cells of the cerebellum, and gray matter (most notably in presumed large motor neurons) of the spinal cord (Fig. 2, *middle*). One shared property of these neuronal populations is their ability to undergo synaptic changes throughout adulthood.

During defined periods of rodent embryonic development, subpopulations of sensory neurons require distinct neurotrophins and their cognate receptors for survival (Pinon et al., 1996). The neurotrophin receptor system remains functional through adulthood. Correspondingly, ARMS expression coincides with that of the Trks and p75. As shown by Northern analysis (Fig. 2, *top*) and *in situ* hybridization (Fig. 2, *bottom*), ARMS expression persists in the adult DRG. In addition to an absence of silver grains over the nerve fibers, there was a notable absence of ARMS message in a subset of DRG cell bodies corresponding to large-diameter neurons. Although ARMS mRNA can be detected in all populations of DRG neurons, much lower expression in large-diameter neurons suggests a less prominent role in proprioception.

More striking was the restricted expression of ARMS during development. In general, during rat nervous system development, the period of embryonic growth between E11 and E14 is a time of massive proliferation of the neuroepithelium. By E14, the first sets of postmitotic neurons are undergoing differentiation. In E14 rat embryos, ARMS was expressed in both spinal cord and dorsal root ganglia (Fig. 3, *left*). These neuronal populations are among the first in the nervous system to undergo differentiation, and it is during this window of development that these postmitotic neurons are actively seeking and making connections with their targets. Additionally, several neuroanatomical loci of the developing brain expressed ARMS mRNA. In the midsagittal plane, the hippocampus, cortex, pons, and medulla were positive for ARMS expression (Fig. 3, *middle*). Regions lateral to the midline such as the basal telencephalon, superior and inferior colliculus, principal trigeminal sensory nucleus, and multiple ganglia, including trigeminal, geniculate, vestibular, and superior cervical (Fig. 3, *right*), showed significant levels of ARMS transcripts.

Association of ARMS with NGF receptors

Because the ARMS protein was originally identified in a yeast two-hybrid assay from its association with the cytoplasmic domain of the p75 receptor, we tested whether ARMS was capable of an interaction with p75 in cultured cells. We cotransfected an HA-tagged p75 cDNA and the ARMS cDNA in HEK293T cells. After immunoprecipitation with an antibody (892) made against the C-terminal region of the ARMS protein, the p75 receptor could be readily detected by Western analysis (Fig. 4*A*). This transfection experiment indicated that ARMS and p75 are capable of forming a complex in HEK293T cells.

Previous studies indicated that p75 and the TrkA receptor can exist in a complex (Huber and Chao, 1995; Gargano et al., 1997; Bibel et al., 1999). To establish whether an interaction between TrkA and ARMS occurs, we performed an immunoprecipitation experiment in PC12 cells expressing elevated levels of TrkA (Hempstead et al., 1992). An NGF-dependent association between ARMS and the TrkA receptor was detected after immunoprecipitation of TrkA and immunoblotting with the anti-ARMS antibody 892 (Fig. 4*B*). The association between ARMS

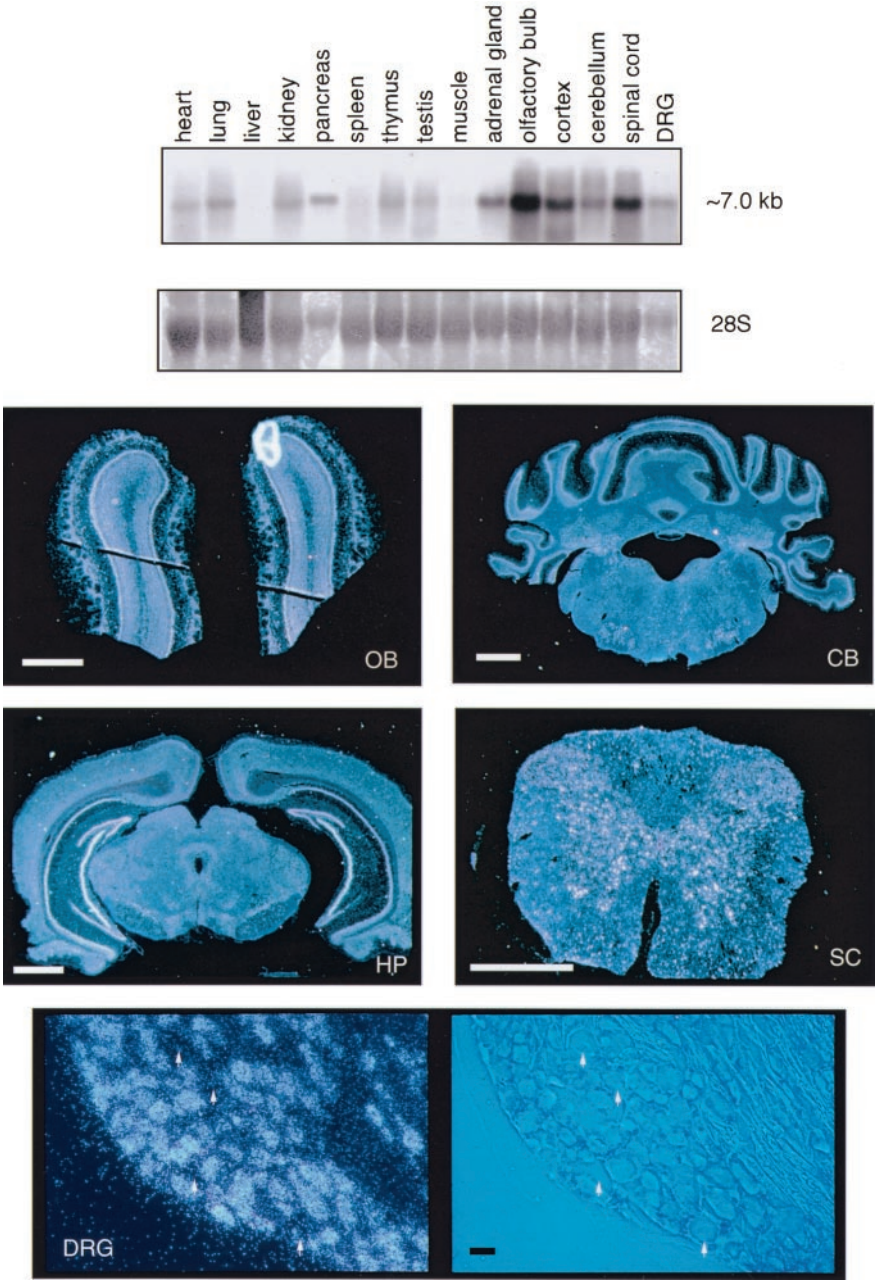

Figure 2. Distribution of ARMS mRNA. *Top*, Northern analysis of ARMS. A single transcript of 7.0 kb was detected by Northern analysis using a ^{32}P-labeled ARMS cDNA probe (*top blot*). Each *lane* contained 20 μg of total RNA (with the exception of the pancreas and DRG *lanes* that contained <10 μg each) extracted from various rat tissue. Methylene blue staining of the 28 S ribosomal band as a loading control is shown (*bottom blot*). *Middle*, Distribution of ARMS mRNA in the adult rat CNS. A ^{33}P-labeled cRNA probe was used to assess ARMS mRNA expression. Areas of intense labeling include the mitral cell layer of the olfactory bulb (*OB*), all regions of the hippocampus (*HP*), the Purkinje cell layer of the cerebellum (*CB*), and gray matter (most notably in the ventral horn) of the spinal cord (*SC*). *Bottom*, Expression in adult rat DRG by *in situ* hybridization. A ^{33}P-labeled cRNA probe was used to assess mRNA distribution in DRG as depicted in the dark-field image (*left*). The majority of cell bodies of the DRG were positive for ARMS mRNA expression, but absences of expression were noted in the large-diameter DRG cell bodies depicted by the *arrows* in the dark-field and the corresponding phase (*right*) photographs. Scale bars: *white*, 1 mm; *black*, 50 μm.

and TrkA persisted 25 hr after NGF treatment. The endogenous association of TrkA and ARMS indicates that ARMS may exist in a ligand-dependent complex with Trk receptors.

To investigate further the distribution and potential colocalization of ARMS and Trk in neuronal cells, we performed indirect immunofluorescence experiments in primary cultures of rat sympathetic neurons. Using the 892 antibody against ARMS and a monoclonal antibody directed against Trk, we observed colocalization of ARMS and TrkA on the cell surface of sympathetic neurons (Fig. 4*C*, *arrow*). These results support our coimmunoprecipitation studies that suggest a physical association between Trk and ARMS.

Tyrosine phosphorylation of ARMS by NGF

The high degree of correspondence in the expression of ARMS and neurotrophin receptors and the endogenous association with TrkA receptors led us to postulate that ARMS might function as a target for Trk receptor phosphorylation. To investigate this possibility, we prepared cell lysates from NGF-treated PC12 cells and immunoprecipitated the ARMS protein with anti-ARMS antiserum 892. Phosphorylation of ARMS protein was visualized by immunoblotting with an anti-phosphotyrosine antibody, pY99. Tyrosine phosphorylation of ARMS was detectable within a minute of NGF addition (Fig. 5*A*, *top*), closely following the time course of TrkA autophosphorylation (Fig. 5*A*, *bottom*). With continued NGF treatment, the phosphorylation of ARMS persisted for 25 hr (Fig. 5*B*). Pretreatment with the alkaloid-like compound K252a (100 nM), which specifically inhibits NGF-mediated activity by selectively blocking the kinase activity of TrkA (Koizumi et al., 1988; Berg et al., 1992), completely abolished the tyrosine phosphorylation of ARMS. Hence, the ARMS protein represents a novel downstream target for the TrkA receptor tyrosine kinase.

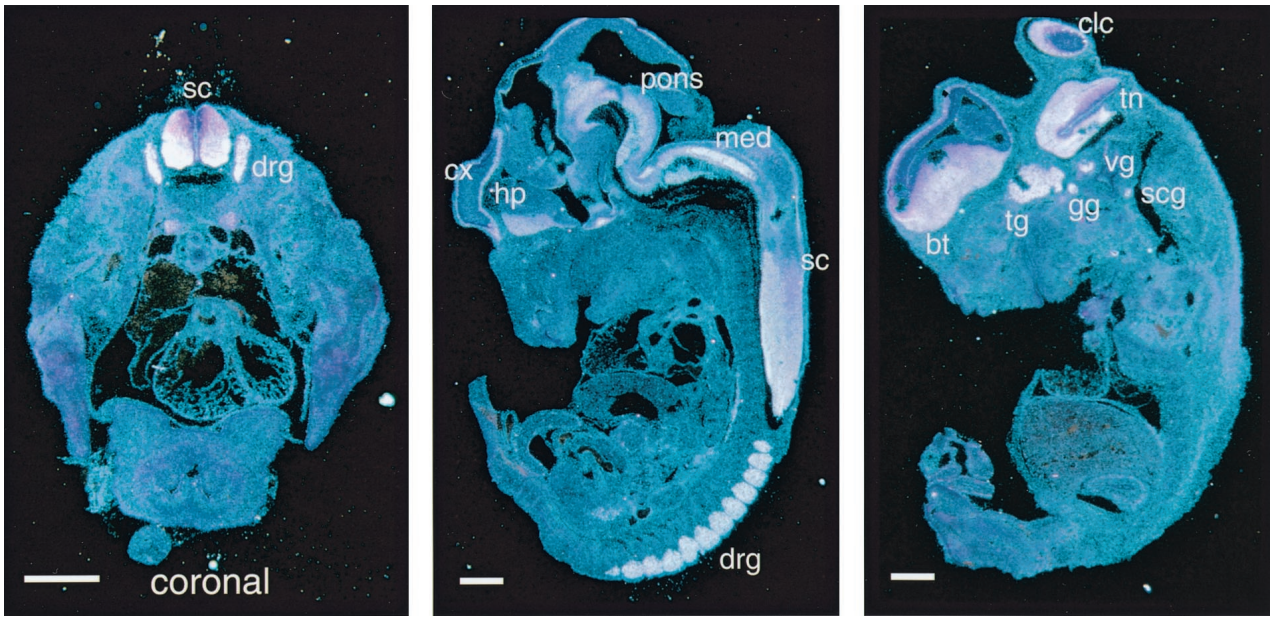

Figure 3. Expression of ARMS mRNA. *In situ* hybridization of ARMS in E14 rat. *Left*, In a coronal section through the midsection of an E14 rat, only spinal cord (*sc*) and dorsal root ganglion (*drg*) were positive for ARMS. *Middle*, *Right*, *In situ* hybridization of ARMS in a midsagittal section and a more lateral section, respectively, of E14 rat is shown. ARMS mRNA expression was restricted to various brain regions such as the cortex (*cx*), hippocampus (*hp*), pons, medulla (*med*), basal telencephalon (*bt*), principal and spinal trigeminal nucleus (*tn*), superior and inferior colliculus (*clc*), and *sc*. Multiple ganglia expressed ARMS mRNA, such as the *drg*, trigeminal ganglion (*tg*), geniculate ganglion (*gg*), vestibular ganglion (*vg*), and superior cervical ganglion (*scg*). Scale bars, 1 mm.

To explore the specificity of ARMS phosphorylation, we compared the effects of EGF and NGF in PC12 cells. The phosphorylation of ARMS was considerably more robust after NGF treatment (Fig. 6), even though both TrkA and EGF receptors were activated. These data indicate that ARMS phosphorylation was a specific consequence of NGF, but not EGF, receptor signaling. This may be a consequence of a lack of association of ARMS with the EGF receptor. It is unlikely that ARMS phosphorylation was mediated via the MAP kinase pathway, because treatment of PC12 cells with EGF showed minimal tyrosine phosphorylation of ARMS after 10 min or 2 hr (Fig. 6). Also, ARMS phosphorylation was not elicited by other growth factors, such as insulin, FGF, and insulin growth factor-1, in PC12 cells (data not shown), indicating that unique proteins are phosphorylated by the receptor tyrosine kinase TrkA.

Response to other trophic factors and ephrins

To test the ability of other Trk receptors to phosphorylate ARMS, we used PC12 cells stably expressing the TrkB receptor. After treatment of these cells with BDNF (100 ng/ml), ARMS was tyrosine phosphorylated, demonstrating that ARMS can be phosphorylated by TrkB as well as TrkA (Fig. 7). That expression of ARMS is localized to some central regions in which TrkA is not expressed, such as the hippocampus, can be reconciled by the presence of TrkB, which is widely expressed in the adult CNS and which serves as a receptor for BDNF and NT-4/5 (Farinas et al., 1998).

To verify the ability of BDNF to induce phosphorylation of ARMS in neurons, primary cultures of hippocampal neurons were established. Hippocampal neurons express the TrkB receptor. After treatment with BDNF, ARMS was immunoprecipitated from hippocampal lysates and assessed for tyrosine phosphorylation by immunoblotting with an anti-phosphotyrosine antibody. A similar phosphorylation of ARMS after BDNF treatment in hippocampal neurons was observed (Fig. 8). Thus, in a primary neuronal culture, ARMS is a downstream target of TrkB after activation by BDNF.

The expression of ARMS during developmental periods of axon outgrowth and synaptogenesis raises the possibility that ARMS is downstream of other known axon guidance regulators. We therefore examined the ephrin family because of its well established *in vitro* and *in vivo* guidance function. As seen in Figure 9*A*, the ARMS protein is potently tyrosine phosphorylated by ephrin B2 in a neuronal/glioma hybridoma cell line, NG108-15, that stably expresses EphB2 receptor. EphB2 receptor autophosphorylation and activation occur over a time period of 30–40 min (Holland et al., 1997), in contrast to a much more rapid activation for Trks. Significantly, the phosphorylation of ARMS followed a time course similar to the time course of Eph receptor autophosphorylation (Fig. 9*B*).

Unlike the Trk receptors, which are activated after dimerization by their respective ligands, Eph receptors are aggregated into multimeric complexes to be biologically active. This differential property of receptor aggregation may account for the differential time courses observed between neurotrophins and ephrins. Interestingly, the time course of TrkA and EphB2 phosphorylation of ARMS suggests that ARMS may be phosphorylated directly by the kinase domains of the receptors themselves and not via downstream intermediates. The integral membrane nature of ARMS and its potentially close proximity to receptor tyrosine kinases could facilitate such a process. Most important, these results suggest that ARMS is phosphorylated by receptor tyrosine kinases with established roles in axonal targeting and guidance. It remains to be determined whether ephrin–Eph clustering and subsequent ARMS phosphorylation are involved in attractive or repulsive effects during axon guidance.

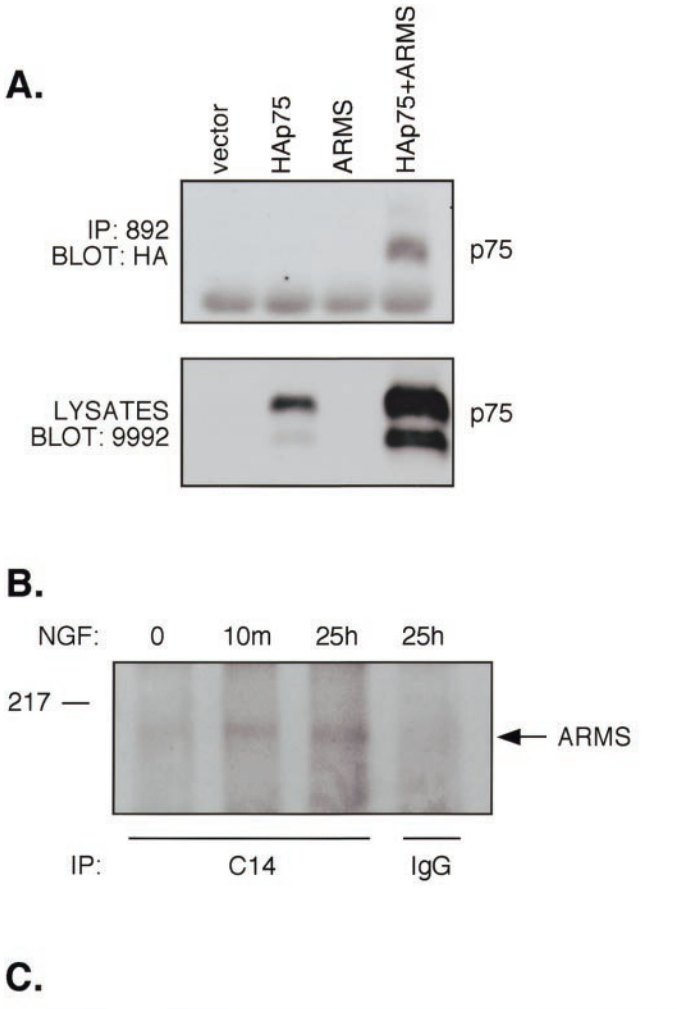

Figure 4. Association of ARMS with p75 and TrkA receptors. *A*, Interaction of p75 with ARMS. HEK293T cells were cotransfected with cDNAs encoding full-length ARMS (*ARMS*), HA-tagged p75 (*HAp75*), ARMS plus p75 (*HAp75+ARMS*), or empty vector (*vector*). Cells lysates were immunoprecipitated with anti-ARMS 892 antiserum and immunoblotted with anti-HA (*top*). Expression of p75 receptors was confirmed by immunoblotting with anti-p75 (9992; *bottom*). *B*, Coprecipitation of TrkA and ARMS. PC12 615 cells were treated for 10 min (*m*) and 25 hr (*h*) with NGF (100 ng/ml). Lysates were prepared and subjected to immunoprecipitation with anti-Trk C14 antibody, followed by immunoblotting with anti-ARMS antibody. Normal rabbit IgG was used as a negative control. The migration of the protein molecular weight standard, 217 kDa, is shown on the *left*. *C*, Colocalization of ARMS and TrkA. Immunofluorescence analysis of ARMS and TrkA receptor in sympathetic neurons is shown. SCG sympathetic neurons were grown in the presence of 150 ng/ml NGF, fixed, and immunostained as described in Materials and Methods. The ARMS protein and the TrkA receptor were subjected to double immunostaining using an anti-ARMS antiserum (*left*) and an anti-Trk B-3 monoclonal antibody (*middle*) and were analyzed by confocal microscopy. The *yellow signal* demonstrates overlap of the two signals (*overlay; right*). The *arrow* indicates cell surface colocalization of ARMS and TrkA. *IP*, Immunoprecipitation.

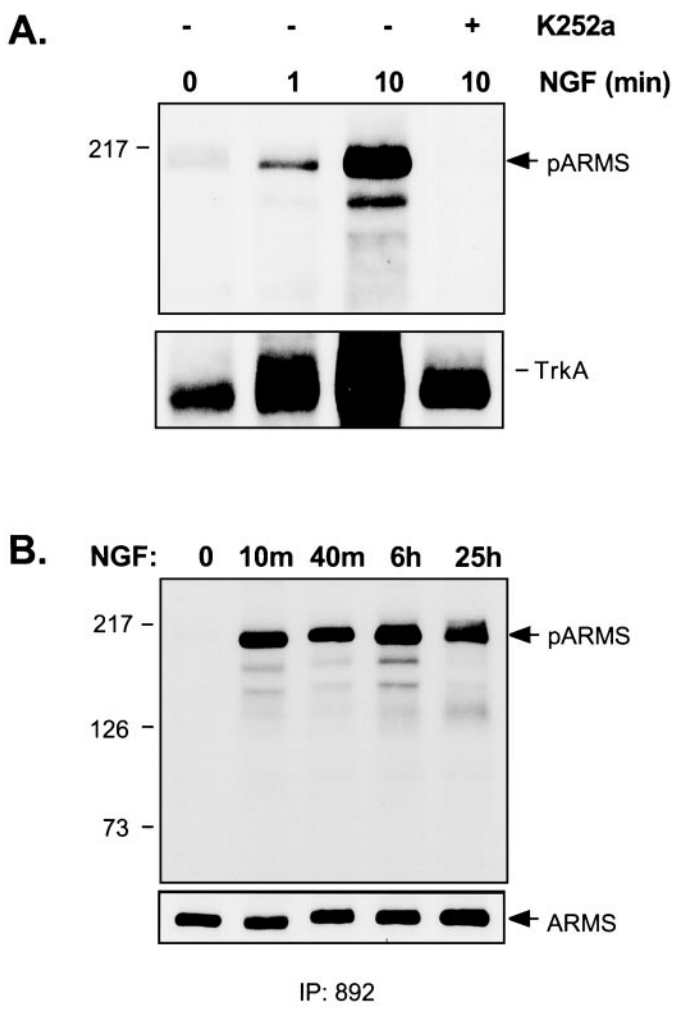

Figure 5. Tyrosine phosphorylation of ARMS. *A*, Phosphorylation of ARMS by NGF in PC12 cells is rapid and can be blocked by K252a. The antiserum 892 was used to immunoprecipitate endogenously expressed ARMS from PC12 615 cell lysates. Anti-phosphotyrosine antibody pY99 was used to assess tyrosine phosphorylation of the immunoprecipitated ARMS. Within 1 min of NGF treatment, phosphorylated ARMS (*pARMS*) could be detected, suggesting a direct phosphorylation by TrkA. Furthermore, 100 nM K252a potently blocked ARMS phosphorylation (*top*). In lysates of the same samples, TrkA autophosphorylation is shown using pY99 (*bottom*). *B*, Time course of ARMS phosphorylation by NGF in PC12 cells is shown. The phosphorylation peaks within 10 min and is sustained for at least 25 hr (*top*). Reprobing of the same blot with 892 demonstrated equivalent levels of immunoprecipitated ARMS from the various lysates (*bottom*).

DISCUSSION

ARMS: a novel downstream target for receptor tyrosine kinases

Although the signaling properties of the Trk receptor tyrosine kinases have been studied extensively, there remain many neurotrophin-stimulated activities in which molecular mechanisms have not been fully defined. These include internalization and transport of receptors, growth cone guidance, and axonal and dendritic branching. A number of common substrates, including phospholipase C-γ, PI-3 kinase, and Shc and Grb2 adaptor proteins, are used by many receptor tyrosine kinases, raising the question of how phosphorylation events lead to different biological outcomes (Chao, 1992b). One possibility is that unique sub-

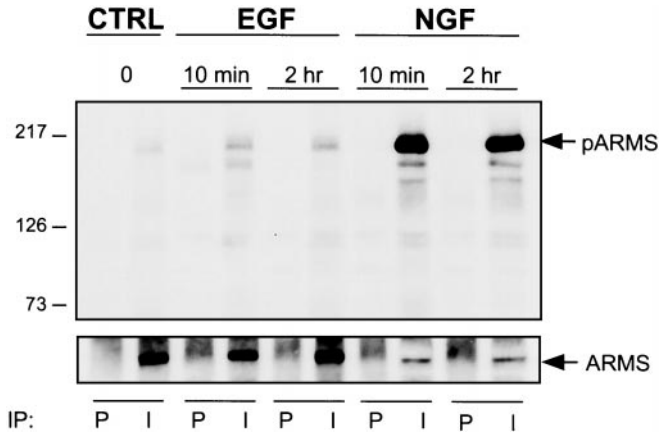

Figure 6. Specificity of ARMS phosphorylation. *Top*, Phosphorylation of ARMS is specifically induced after NGF, but not EGF, treatment of PC12 615 cells. Two time points, 10 min and 2 hr, were examined for tyrosine phosphorylation of ARMS using the following conditions: no ligand (*CTRL*), 50 ng/ml EGF, and 100 ng/ml NGF. To demonstrate the specificity of the ARMS antiserum 892 (*I*), preimmune antiserum (*P*) was used in parallel immunoprecipitations. *Bottom*, The amount of ARMS protein that was immunoprecipitated from the various lysates is shown.

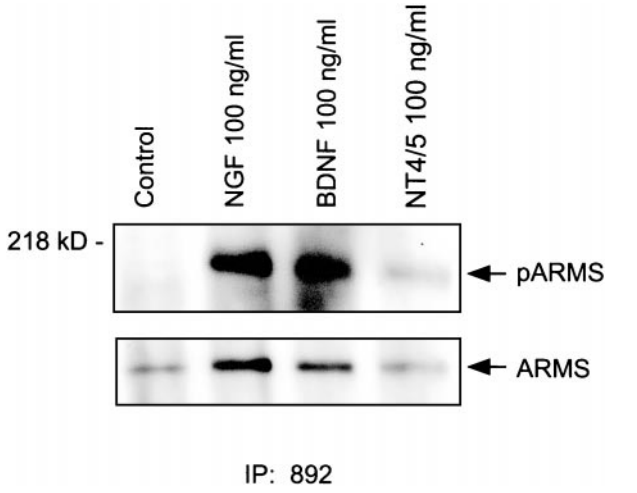

Figure 7. Phosphorylation of ARMS by other neurotrophins. The neurotrophins BDNF and NT-4/5 induce phosphorylation of ARMS via the TrkB receptor. PC12 cells stably expressing TrkB were treated with either 100 ng/ml BDNF or 100 ng/ml NT-4/5, and the phosphorylation of ARMS was measured as described in Figure 5. *Top*, BDNF and, to a lesser extent, NT-4/5 were able to induce tyrosine phosphorylation of ARMS. *Bottom*, Immunoprecipitated ARMS from each lysate is shown.

strates exist that determine the specific nature of neurotrophin responses.

Here we describe the properties of a novel tyrosine-phosphorylated transmembrane protein. The ARMS protein is highly expressed in many neuronal populations and functions as a downstream target for both Trks and Eph receptors. A common feature of ARMS, p75, TrkA, and Eph receptors is that they all contain putative C-terminal PDZ-binding motifs. It is therefore possible that these proteins are localized to the same subcellular compartment by PDZ domain-containing molecules, and this localization may contribute to signaling events both developmentally and in adulthood. Indeed, the EphB2 receptor has been found to be associated with PDZ-containing proteins, and this association is thought to be important for receptor function at

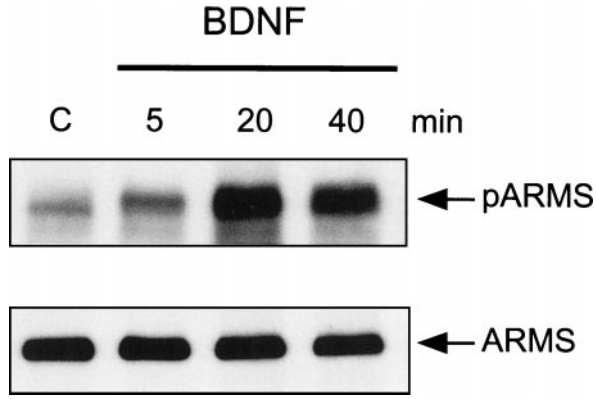

Figure 8. Induction of ARMS phosphorylation in hippocampal neurons by BDNF. Primary cultures of E17 hippocampal neurons were prepared and treated with 50 ng/ml BDNF for the indicated times. *Top*, Phosphorylation of ARMS was assessed by immunoprecipitation with anti-ARMS 892 antiserum and Western blotting with anti-phosphotyrosine pY99 antibody. *Bottom*, Equal amounts of ARMS protein were immunoprecipitated from each lysate as shown by reprobing the same blot with 892. *C*, Control.

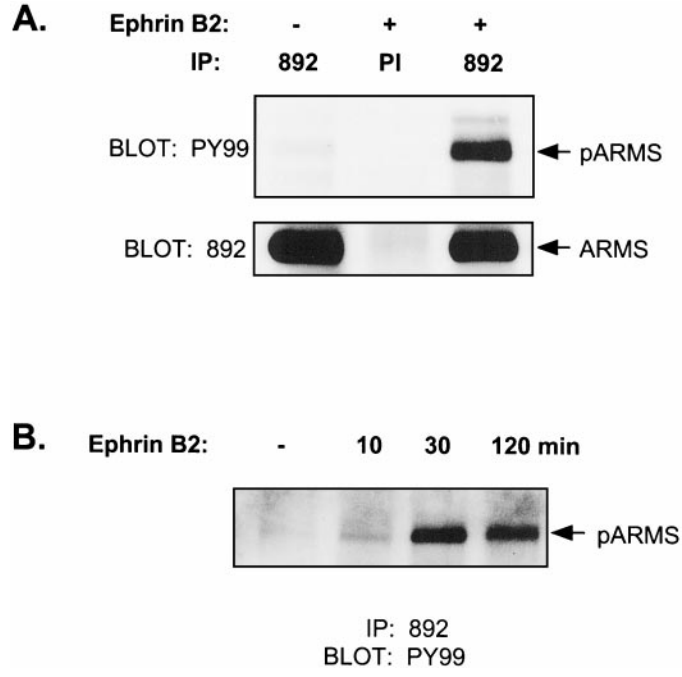

Figure 9. Phosphorylation of ARMS by ephrins. *A*, Ephrin B2 induces ARMS tyrosine phosphorylation in NG108-15 cells expressing EphB2 receptor. Lysates were made from untreated or ligand-stimulated NG108-15 cells (using aggregated ephrin B2; 30–40 min) and immunoprecipitated with 892 antiserum or preimmune (*PI*) serum. Tyrosine phosphorylation was assessed with pY99 in subsequent Western blots (*top*). Equivalent amounts of ARMS were immunoprecipitated (*bottom*). *B*, Tyrosine phosphorylation of ARMS by ephrin B2 peaks at 30 min. Thus, the time course of ARMS tyrosine phosphorylation closely parallels that of receptor autophosphorylation.

neuronal synapses (Torres et al., 1998) and for vestibular axon guidance and ionic homeostasis in the inner ear (Cowan et al., 2000).

At E14, trigeminal and vestibular axons migrate toward their peripheral targets and commence synapse formation. High levels of ARMS mRNA expression in these subpopulations suggest that ARMS may participate in axonogenesis or axon guidance. A

notable absence of ARMS mRNA expression was observed in germinal zones of the developing brain, regions that are extensively proliferating. A general conclusion that can be drawn from the *in situ* hybridization studies described here is that ARMS-positive neuron populations, for the most part, are postmitotic and postmigratory. For example, the somatic and visceral motor neurons of the spinal cord are born by E12 and E13 but by E14 are already undergoing axonogenesis (Paxinos, 1995). It is during this postmigratory, differentiative stage that ARMS is highly expressed. Conversely, the presumptive olfactory bulb, which is devoid of neurons at this stage of development, is negative for ARMS expression; the absence of ARMS expression in premigratory neuronal populations supports the observation that ARMS expression may be restricted to postmitotic and postmigratory neurons. These findings suggest that ARMS may be involved in postmigratory events, such as axon guidance or synaptogenesis.

We observed expression of ARMS in the adult CNS in regions such as the olfactory bulb, hippocampus, Purkinje cells of the cerebellum, and spinal cord motor neurons. The most significant common attribute of these neuronal populations is their ability to undergo continued synaptic changes throughout adult life. Neurons of the olfactory bulb are continually renewed and hence must form new synapses, hippocampal neurons can undergo synaptic remodeling and long-term potentiation, Purkinje cell dendrites are highly plastic because of their constant structural remodeling, and motor neurons have the capacity to regenerate and to form new synapses with peripheral targets in adults. Peripheral neurons have regenerative properties throughout adulthood, and this process requires axon outgrowth and new synapse formation. Neurotrophins and ephrins are likely candidates in spinal cord regeneration (Frisén et al., 1992; Miranda et al., 1999), and it remains to be determined whether ARMS is also used in this process.

Colocalization of ARMS with neurotrophin and ephrin receptors

The *in vivo* use of ARMS by the neurotrophin and ephrin receptors is supported by coexpression of these proteins during development. For example, Trk and p75 expression can be detected in all peripheral ganglia of neural crest origin (e.g., superior cervical and dorsal root), spinal cord, and brainstem, regions also high in ARMS mRNA (Yan and Johnson, 1988; Snider, 1994). In the adult, ARMS and p75 expression overlaps most notably in the olfactory bulb, Purkinje cells of the cerebellum, and motor neurons of the spinal cord. Additionally, Trk receptors are found throughout the nervous system in regions that also express ARMS. For example, TrkA and ARMS are both expressed in the small-diameter sensory neurons of the DRG (Averill et al., 1995; Wright and Snider, 1995). Adult hippocampus, cerebellar Purkinje cells, and motor neurons of the spinal cord are a few regions in which the expression of ARMS and TrkB significantly overlaps (Yan et al., 1997).

In a similar manner, Eph receptor and ARMS expression overlaps in adult hippocampus and spinal motor neurons. During development, the Eph receptors are also found in ganglia of neural crest origin (e.g., sensory and vestibular) and in the tectum (Flanagan and Vanderhaeghen, 1998), regions that also express ARMS. Ephrins and their receptors have a well known function in establishment of the retinotectal pathway, and the expression of ARMS in the tectum suggests that ARMS plays a role in this process.

Because of the multiple cell populations that express Trks, Eph receptors, and ARMS, such as sensory and motor neurons, our findings raise the possibility of cross-talk between these two receptor systems. ARMS may serve to link or modulate these two signaling pathways in a cooperative or competitive manner. This could potentially be achieved by receptor phosphorylation of similar or different tyrosine residues of ARMS and/or association with other membrane proteins. It is plausible that ARMS may serve as an adaptor protein, because of its PDZ-binding motif and other protein interaction domains that would allow for recruitment of multiple, diverse proteins.

Other potential functions

The predicted integral membrane structure of ARMS led us to hypothesize that ARMS may function as an ion channel. The four predicted transmembrane domains of the ARMS protein and its overall structure are reminiscent of the transient receptor potential family of ion channels and the vanilloid (capsaicin) receptor VR1 (Caterina et al., 1997; Harteneck et al., 2000). These channels contain six transmembrane domains with intracellular N and C termini and N-terminal ankyrin repeats. Although we have not detected ARMS channel activity in gene transfer experiments, it remains possible that this protein may also serve as a subunit of a channel or is involved in clustering or maintenance of ion channels. Such a possibility has been suggested by the ability of BDNF to elicit hippocampal, cortical, and cerebellar depolarization on a very rapid time scale (Kafitz et al., 1999), presumably via activation of sodium channels. Although the mechanism of this activation is unknown, phosphorylation of ARMS by TrkB suggests a role for ARMS in neurotrophin-mediated regulation of neuronal activity.

The ARMS protein is a novel, neuron-enriched protein that is highly conserved throughout evolution. ARMS is expressed in postmitotic neurons during the stage of development in which extensive axon pathfinding is occurring and is also expressed during adulthood in "plastic" regions of the brain. The presence of multiple protein interaction domains strongly supports a role for ARMS in recruiting proteins to Trk receptor tyrosine kinases. Indeed, coimmunoprecipitation studies indicate that ARMS may be an integral part of a higher order Trk–p75 receptor complex. These interactions may not be limited to neurotrophin signaling, because the Eph receptor family is also capable of phosphorylating the ARMS protein. Further studies to characterize the proteins that interact with ARMS and the consequences of *ARMS*-targeted deletion will likely help elucidate the role of this evolutionarily conserved protein.

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