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Intersectin, an Adaptor Protein Involved in Clathrin-mediated Endocytosis, Activates Mitogenic Signaling Pathways*

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Anthony Adams‡, Judith M. Thorn‡, Montarop Yamabhai§¶, Brian K. Kay§, and John P. O'Bryan‡‖

From the ‡Laboratory of Signal Transduction, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709 and the §Department of Pharmacology, University of Wisconsin, Madison, Wisconsin 53706

Intersectin is a member of a growing family of adaptor proteins that possess conserved Eps15 homology (EH) domains as well as additional protein recognition motifs. In general, EH domain-containing proteins play an integral role in clathrin-mediated endocytosis. Indeed, intersectin functions in the intermediate stages of clathrin-coated vesicle assembly. However, recent evidence suggests that components of the endocytic machinery also regulate mitogenic signaling pathways. In this report, we provide several lines of evidence that intersectin has the capacity to activate mitogenic signaling pathways. First, intersectin overexpression activated the Elk-1 transcription factor in an MAPK-independent manner. This ability resides within the EH domains, as expression of the tandem EH domains was sufficient to activate Elk-1. Second, intersectin cooperated with epidermal growth factor to potentiate Elk-1 activation; however, a similar level of Elk-1 activation was obtained by expression of the tandem EH domains suggesting that the coiled-coil region and SH3 domains act to regulate the EH domains. Third, intersectin expression was sufficient to induce oncogenic transformation of rodent fibroblasts. And finally, intersectin cooperated with progesterone to accelerate maturation of *Xenopus laevis* oocytes. Together, these data suggest that intersectin links endocytosis with regulation of pathways important for cell growth and differentiation.

EH[1] domain-containing proteins are a growing family of adaptor proteins that play an integral role in clathrin-mediated endocytosis. This idea was first suggested by the finding that mutations in the yeast EH domain-containing proteins Pan1p and End3p have profound effects on endocytosis (1, 2). These proteins are related to the mammalian Eps15 protein and suggest that Eps15 is involved in vesicular transport. Indeed, Eps15 has been localized to the rims of clathrin-coated pits (3) and dominant-negative Eps15 mutants or Eps15 antibodies inhibit clathrin-mediated endocytosis (4, 5). Another group of proteins with EH domains include the intersectin family, which consists of intersectin (frog and human) (6, 7), Dap160 (fruit fly) (8), Ese 1/2 (mouse) (9), and EHSH1 (rat) (10). These related proteins contain two NH_2-terminal EH domains, a central coiled-coil region, and four or five SH3 domains at the COOH terminus.

Accumulating data support the notion that intersectin is a component of the endocytic machinery. First, intersectin localizes to the plasma membrane at clathrin-coated pits (9, 11). Second, intersectin binds components of the endocytic complex, including dynamin, Eps15, and epsin (6, 8, 9, 11). Third, expression of full-length or truncated forms of intersectin inhibit endocytosis of the transferrin receptor (9, 12). Together, these data suggest that intersectin plays an important role as an accessory protein in endocytosis.

Although intersectin is believed to function in clathrin-mediated endocytosis, several lines of evidence suggested to us that this protein might possess an additional function in regulating signaling pathways. First, like many cytoskeletal and signal transduction proteins, intersectin contains SH3 domains, which interact with pro-rich peptide sequences within proteins (13). Second, both *in vitro* and *in vivo* experiments indicated that the SH3 domains of intersectin interact with the Ras exchange factor, Sos (6, 14).[2] Third, splice variants of intersectin specifically expressed in the brain possess an extended COOH terminus consisting of a Dbl homology (DH) domain, a pleckstrin homology (PH) domain, and a C2 domain suggesting an involvement in the regulation of Rho family of small GTP binding proteins and Ca^{+2}-dependent molecular interactions (7, 9, 11). Comparison of the primary structure of the DH/PH domains of intersectin with other Dbl family members suggests that the long form of intersectin may function as an exchange factor for Rho family GTPases.[3] Finally, overexpression of Eps15, an intersectin-related protein that physically associates with intersectin (9), induces oncogenic transformation of rodent fibroblasts, suggesting a potential involvement of EH domain-containing proteins in regulation of mitogenesis (15). In support of this notion, the *Eps15* gene has been found as a reciprocal translocation with the *HRX* gene in rare cases of acute myelogenous leukemias (16). Thus, we examined whether intersectin regulates signal transduction pathways and if these pathways contribute to cellular growth control. Our findings indicate that intersectin activates the

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¶ Present address: Dept. of Cell Biology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235.

‖ To whom correspondence should be addressed: National Institute of Environmental Health Sciences, National Institutes of Health, Bldg. 101, Rm. F336, MD F3–06, P.O. Box 12233, Research Triangle Park, NC 27709. Tel.: 919-541-3619; Fax: 919-541-1898; E-mail: obryan@niehs.nih.gov.

[1] The abbreviations used are: EH, Eps15 homology; SH3, Src homology 3; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; RTK, receptor tyrosine kinase; HA, hemagglutinin, ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; GVBD, germinal vesicle breakdown; DH, Dbl homology; PH, pleckstrin homology; PCR, polymerase chain reaction; RLU, relative light units; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

[2] J. P. O'Bryan, unpublished observations.

[3] K. Rossman and S. Campbell, personal communication.

Elk-1 transcription factor and induces oncogenic transformation of cells. In addition, intersectin cooperates with progesterone to induce the maturation of *Xenopus* oocytes. These data provide evidence that intersectin may have a dual role in the cell: assembly of clathrin-coated vesicles and activation of signal transduction pathways.

EXPERIMENTAL PROCEDURES

Cells and Reagents—Human 293T cells and NIH/3T3 cells were cultured as described previously (17). cDNA constructs encoding hemagglutinin (HA) tagged full-length intersectin and a construct encoding the intersectin EH domains (amino acids 11–306) were prepared by PCR as described previously (11). Briefly, the SH3 construct (amino acids 721–1216) was prepared in an identical manner using the following primers: SH3Fa (5′-GATCCGAGAAAGCCCCTCTAACGATC); SH3Fb (5′-CGAGAAAGCCCCTCTAACGATC); SH3Ra (5′-GATCCCGGTATTCCACCTGCTGGTTT); and SH3Rb (5′-CCGGTATTCCACCTGCTGGTTT). The SH3 domains were prepared by PCR using an intersectin cDNA template with *Pfu* DNA polymerase using the above primers. Two sets of PCR reactions were performed with the following primer pairs: one reaction with primer pairs SH3Fa and SH3Rb and another reaction with primer pairs SH3Fb and SH3Ra. The two reaction products for the SH3 construct were mixed, and the double stranded DNA was denatured and allowed to re-anneal. One of four possible double stranded DNAs in each re-annealed mixture contains sticky ends at both ends, which are compatible for a *Bam*HI site. The re-annealed mixtures were ligated into the pCGN-Hygro vector (18), which carries a *Bam*HI site in-frame with the HA epitope tag. An expression construct encoding the helical region (Coil, amino acids 304–738) was prepared by PCR using *Pfu* DNA polymerase with an intersectin template and the following primers: HelixF (5′-CTGTGCGGATCCCCCCCCATCTTTTAGAAGAGTTCGA) with HelixR (5′-CTGTGCGGATCCTCGGTAATACACAACCTTCACATC). PCR reactions were digested with *Bam*HI and subcloned into the *Bam*HI site in pCGN-Hyg. An expression construct encoding *Xenopus* MP90 (19), was PCR-amplified from an MP90 cDNA using the following primers: MP90F (5′-CTGTGCGGATCCATGAAAAACATAGTCCACAATTAC) and MP90R (5′-CTGTGCGGATCCCCTCTACAAACTCACCGCACCTC). PCR reactions were digested with *Bam*HI and subcloned into the *Bam*HI site in pCGN-Hyg as above. All constructs were confirmed by sequence analysis. Sequencing of the MP90 clone revealed a mutation in the sequence at codon 169 that changes the amino acid from Ser to Pro as compared with that in GenBank™ (accession number U95102). However, comparison of the rat and mouse homologs indicates a Ser at the comparable position. Thus, we believe that our MP90 clone is actually wild type.

Reporter Analysis—Elk-1 activation was measured as described previously (17). Following serum starvation overnight, cells were either left unstimulated or stimulated for 4–6 h with 100 ng/ml EGF. Cells were washed with warm phosphate-buffered saline then harvested in 1× luciferase lysis buffer (Analytical Luminescence). Luciferase activity was then measured in a Dynex 96-well microtiter plate luminometer, and the relative light units (RLU) per microgram of protein were determined. For each experiment, relative activation was determined by dividing the RLU/μg for each experimental point by the RLU/μg for unstimulated vector control. The MEK inhibitor U0126 (Promega) was used at a final concentration of 10 μM in dimethyl sulfoxide.

MAPK Activation—HEK 293T cells were transiently cotransfected with the indicated expression constructs along with an HA epitope-tagged Erk-2 expression construct. On the following day, cells were placed in serum-free media overnight. On the following morning, cells were stimulated with or without EGF (10 ng/ml) for 10 min at 37 °C. *In vitro* MAPK assays were performed on anti-HA immunoprecipitates as described previously (17). Briefly, equal amounts of protein (500 μg) were immunoprecipitated with an HA monoclonal antibody (Babco). Precipitates were washed two to three times with ice-cold PLC-LB (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton-X-100, 1 mM EGTA, 1.5 mM magnesium chloride, 100 mM sodium fluoride) containing inhibitors then washed two times with kinase buffer (20). Pellets were resuspended in 37 μl of kinase buffer containing [γ-^{32}P]ATP and then incubated at 30 °C for 20 min. Reactions were stopped with the addition of 50 μl of 2× SDS Laemmli sample buffer. After heating to 100 °C for 10 min, samples were fractionated by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P membranes, and allowed to dry. The amount of ^{32}P incorporation into the myelin basic protein band was determined by densitometric analysis. The remainder of the filters were subjected to Western blot analysis with the anti-HA antibody to determine the levels of MAPK expression using enhanced chemiluminescence. Signals were quantitated by densitometry of x-ray films.

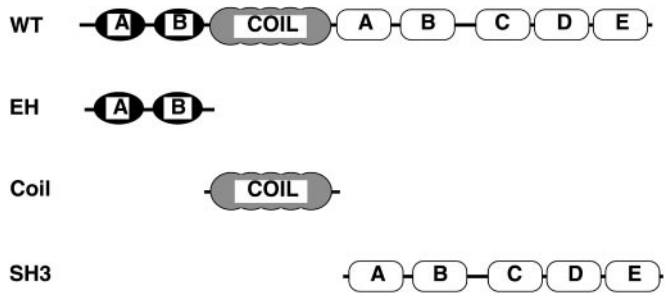

FIG. 1. **Intersectin expression constructs.** Each construct was subcloned into pCGN-Hyg expression vector, which encodes a hemagglutinin epitope sequence at the 5′-end of the subcloned cDNA fragments. *WT*, wild type intersectin includes amino acids 1–1278; *EH*, encodes both EH domains from amino acids 11–306; *Coil*, encodes the region of α-helical structure from amino acids 304–738; *SH3*, encodes all five SH3 domains from amino acids 721–1216.

Transformation Assays—NIH/3T3 cells were plated at a density of 2×10^5 cells per 60-mm tissue culture dish in Dulbecco's modified essential media supplemented with 10% calf serum, 100 units/ml penicillin/streptomycin and 110 mg/liter sodium pyruvate. Cells were transfected with 2 μg of DNA as described previously (21). Transfected cells were grown for 3–4 days, trypsinized, and replated in 100-mm dishes at a 1:10 dilution of the harvested volume. Cells were initially selected in complete media containing 200 μg/ml hygromycin B (Roche Molecular Biochemicals) then moved to 400 μg/ml hygromycin B after a few days. Once selected, colonies were pooled, passaged, and allowed to sit at confluence for approximately 18 days whereupon they were fixed in 30% methanol/10% acetic acid then stained with a 1% crystal violet solution. For clonal analysis, individual colonies were picked, expanded, and then tested for focus forming activity as above.

Oocyte Maturation Assays—Intersectin coding sequences were PCR-amplified from the mammalian expression constructs using primers that incorporate an SP6 promoter to drive transcription *in vitro* and add a poly(A)+ tail at the 3′-end of the PCR product. The sequence of the primers is as follows: 5′-SP6HA (5′-GAGAATTTAGGTGACACTATAGAACTCACCATGGCTTCTAGCTATCCTTAT); 3′-poly(A) (5′-GAGATTTTTTTTTTTCACCCTGAAGTTCTCAGGAT). Sense mRNA was synthesized from the PCR products using SP6 RNA polymerase mMESSAGE mMACHINE (Ambion). Segments of adult ovary were removed surgically from adult females (*Xenopus*1, Dexter, MI) and incubated in 0.1% collagenase D (Roche Molecular Biochemicals) in OR-2 (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 1.0 mM Na_2HPO_4, 5.0 mM HEPES, 3.8 mM NaOH, pH 7.6). The dissociated oocytes were then washed three to five times with fresh OR-2 and allowed to recover overnight at 18 °C. On the following day, stage VI oocytes were selected and injected with mRNA (1–10 ng) or water (mock) and incubated overnight at 18 °C. For each experiment, oocytes were treated with progesterone (0.5–2 μg/ml) (Sigma) and scored visually for germinal vesicle breakdown (GVBD) every 30 min.

RESULTS

Intersectin Activates Elk-1—To assess the potential role of intersectin in regulating signal transduction cascades, we tested a series of expression constructs encoding *Xenopus* intersectin (Fig. 1). When transfected into 293T cells, each of the three truncation mutants was expressed at similar levels, although expression of full-length intersectin was significantly lower than the truncation mutants (data not shown). We then tested the ability of these intersectin molecules to activate signal transduction pathways. Using a transient luciferase reporter assay, we found that expression of full-length intersectin led to potent activation of the Elk-1 transcription factor (Fig. 2*A*). The levels of intersectin activation of Elk-1 was approximately 2-fold greater than the level obtained with maximal EGF stimulation. To determine the structural basis for this activity, we expressed various intersectin truncation mutants and assessed their ability to activate Elk-1. As shown in Fig. 2*A*, the ability of intersectin to activate Elk-1 resides within the EH domains. Furthermore, expression of the tandem NH_2-

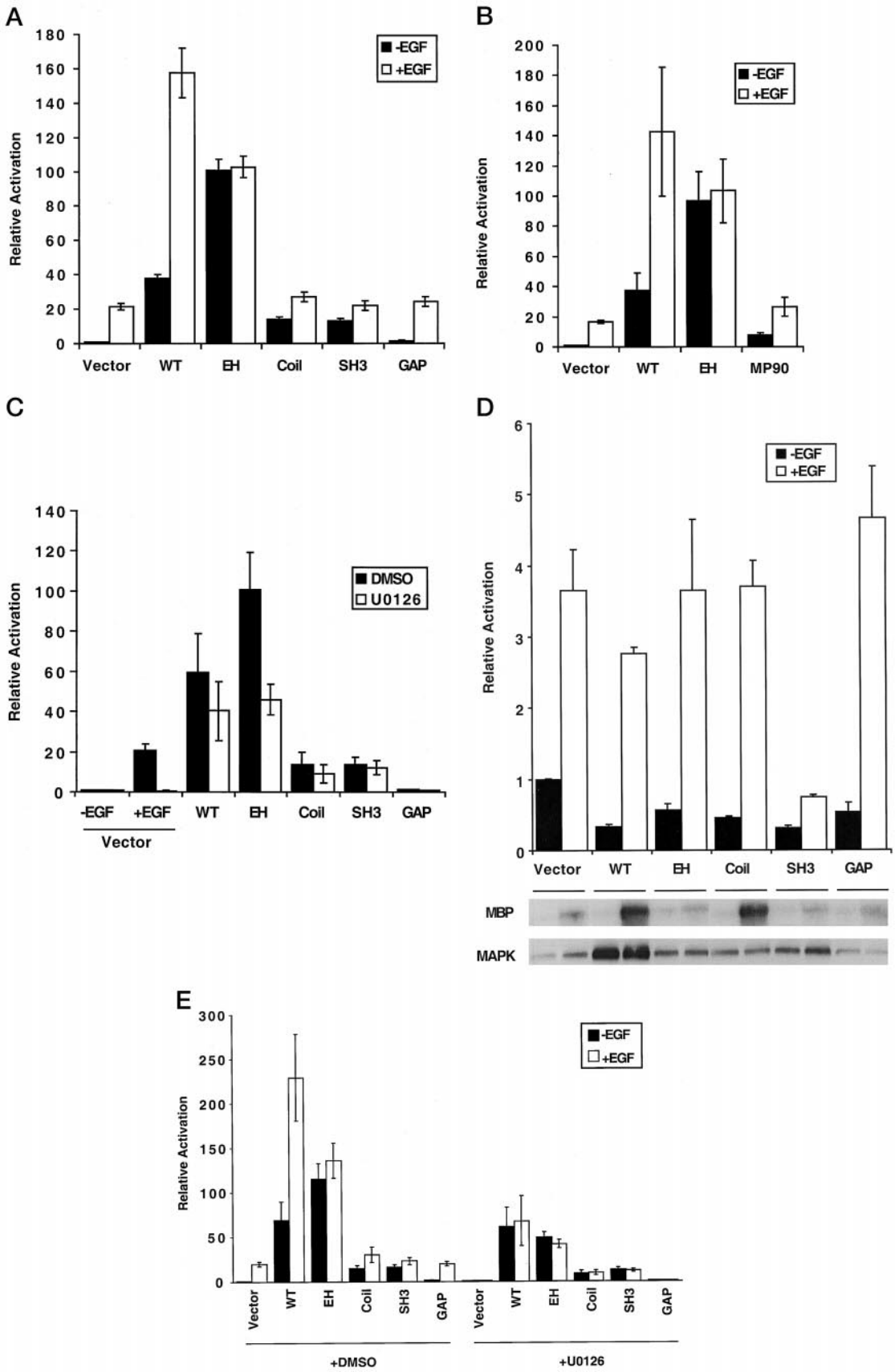

FIG. 2. **Intersectin expression leads to Elk-1 activation.** HEK-293T cells were used to measure Elk-1 activation as described previously (17). *A*, intersectin activates Elk-1 in both a growth factor-independent and -dependent manner. Results are expressed as -fold activation relative to the unstimulated vector control (pCGN-Hyg). Data are expressed as the mean ± S.E. for five independent experiments performed in duplicate. *B*, MP90 does not mediate intersectin activation of Elk-1. Results are expressed in the same way as in *A*. Data are expressed as the mean ± S.E. for three independent experiments performed in duplicate. *C*, intersectin activation of Elk-1 is refractory to MEK inhibition. Same as in *A* except cells were treated overnight with either vehicle control (dimethyl sulfoxide) or the MEK inhibitor U0126 (10 μM) and then processed as in *A*. Data are

terminal EH domains led to an even greater level of activation of Elk-1 than expression of the full-length protein. These results suggest that the ability of intersectin to activate Elk-1 resides within this 296-amino acid segment and, furthermore, that the presence of the coiled-coil region and SH3 domains represses intersectin's ability to activate Elk-1.

Because Elk-1 is a target of numerous growth factor signaling pathways, we next tested whether intersectin might cooperate with growth factor-activated pathways to activate Elk-1. Interestingly, stimulation of cells overexpressing intersectin with epidermal growth factor (EGF) led to a synergistic enhancement of Elk-1 activation, implying that cross-talk can occur between intersectin and receptor tyrosine kinases (RTKs) (Fig. 2*A*). As mentioned above, expression of the tandem EH domains partially mimicked this cooperativity, suggesting that growth factor stimulation suppresses the negative regulation by the coiled-coil and SH3 regions. Thus, interaction of intersectin with additional cellular proteins, either through the coiled-coil region and/or the SH3 domains, may serve to regulate the function of the EH domains. Although the coiled-coil region and SH3 domains did weakly activate Elk-1 (13- to 14-fold) by themselves, we did not observe the same level of Elk-1 activation as seen with the full-length or EH region nor did we observe synergism between these domains and growth factor signaling pathways. These data indicate that intersectin activates signal transduction pathways leading to Elk-1 activation. Furthermore, intersectin appears to cooperate with the EGFR signaling pathway to potentiate Elk-1 activation.

The EH domains of intersectin and Eps15 physically interact with epsin, another component of the endocytic machinery. Given the ability of the EH domains of intersectin to stimulate Elk-1, we tested whether this activity was due to the interaction with epsin, an EH binding protein involved in endocytosis (22). Expression of MP90 (19), the *Xenopus* homolog of epsin, led to proper targeting of the protein to clathrin-coated pits and suggested that the protein was functional (data not shown). When tested in the Elk-1 transcriptional activation assay, MP90 expression lead to a slight activation of Elk-1 (~7-fold); however, there was no cooperative effect on EGF activation of Elk-1 (Fig. 2*B*). Interestingly, MP90 was expressed at approximately 2-fold higher levels than was the EH domains, suggesting that the ability of intersectin to activate Elk-1 is specific and is not due to its interaction with the epsin family of proteins.

Intersectin Activation of Elk-1 Is Independent of p42/p44 Erks—Given that Elk-1 is an important target for a variety of signaling pathways, we have begun to examine the mechanism for intersectin activation of Elk-1. Because Elk-1 is activated by the p42/p44 Erk family of MAPKs in many cell types, we tested whether intersectin activated Elk-1 through activation of Erks. Although treatment of cells with U0126, a pharmacological inhibitor of the p42/p44 Erk activators MEK-1 and -2 (23), completely abolished EGF activation of Elk-1, intersectin activation of Elk-1 was refractory to treatment (Fig. 2*D*). To confirm this result, we examined whether intersectin activated p42/p44 Erk-2 by measuring the *in vitro* kinase activity of a hemagglutinin (HA) epitope-tagged p42Erk-2 in cells overexpressing the various intersectin proteins. As shown in Fig. 2*D*, stimulation of control cells (vector) with EGF led to activation of p42Erk-2. In addition, expression of a constitutively activated form of Ras (H-Ras61L) also led to potent p42Erk-2 activation (data not shown). In contrast, expression of intersectin neither induced Erk-2 activation nor altered Erk-2 activation by EGF. These data support the conclusion that intersectin activation of Elk-1 occurs independently of the p42/p44 Erk-MAPK pathway.

Cooperativity between Intersectin and EGFR Requires the Erk-MAPK Pathway—Although intersectin did not activate Erk-MAPKs, we tested whether the synergistic enhancement of Elk-1 activation by EGFR and intersectin required Erk-MAPK activity. As shown in Fig. 2*E*, inhibition of Erk activation with U0126 abolished the cooperative interaction between EGFR and intersectin. These results suggest that intersectin-activated pathways, together with the EGFR-activated Erk pathway, cooperate to activate transcription factors such as Elk-1.

Intersectin Expression Induces Oncogenic Transformation of Rodent Fibroblasts—Given the ability of intersectin to activate signaling pathways such as Elk-1, we tested whether intersectin might also regulate mitogenic pathways. Overexpression of intersectin in rodent fibroblasts induced the formation of morphologically transformed cells (Fig. 3). Analysis of clonal cell lines indicated that the degree of transformation correlated with the levels of intersectin protein in the cells suggesting that transformation was due to overexpression of wild type intersectin (Fig. 3, *A* and *B*). Unlike oncogenic Ras, which is a potently transforming gene, intersectin's transforming activity was quite weak. Furthermore, the appearance of the intersectin foci was distinct from Ras-induced foci. Together these data suggest that intersectin activates a distinct signaling pathway(s) from Ras in agreement with the observed lack of MAPK activation by intersectin (above). Thus, in addition to activating transcriptional pathways, intersectin also promotes growth transformation of cells.

To determine the region of intersectin important for transformation, we tested the ability of the intersectin mutant constructs to induce morphological transformation. As shown in Fig. 3*C*, expression of either full-length intersectin or the EH domain region was sufficient to induce transformation of rodent fibroblasts. The SH3 region, however, was not capable of inducing transformation. These results suggest that intersectin-induced transformation occurs through the EH region. Although the coiled-coil domain did not appear to induce transformation as illustrated in Fig. 3*C*, we could sometimes detect sporadic transformation in cells expressing this domain; however, because transformation was not consistently detected, we do not believe that this region possesses oncogenic activity.

Intersectin's SH3 Domains Inhibit EGFR-mediated Activation of MAPK—Accumulating evidence suggests that certain RTKs and G protein-coupled receptors require endocytosis to activate the MAPK pathway (24, 25). For example, EGFR activation of MAPK is inhibited by a mutant version of dynamin that blocks endocytosis (25). Recently, the SH3 domains of intersectin were shown to block endocytosis (12). Given the involvement of intersectin in endocytosis, along with our findings that intersectin synergizes with the EGFR to activate Elk-1 and induce transformation, we tested whether intersectin might serve as a potential link between RTKs and the endocytic pathway. Expression of a truncation mutant of intersectin consisting of the isolated SH3 region partially inhibited EGFR-induced activation of MAPK, whereas expression of the SH3 domain from the Ras GTPase accelerating protein (p120RasGAP) had no affect on EGFR activation of MAPK (Fig.

expressed as the mean ± S.E. for four independent experiments performed in duplicate (five experiments for GAP SH3). *D*, intersectin regulates EGFR activation of p42Erk MAPK. Results are expressed as the amount of ^{32}P incorporated per level of Erk-2 expression. The levels of relative activation are compared with the value for vector-transfected cells without growth factor stimulation. Data are expressed as the mean ± S.E. for three independent experiments. Shown below the graph is a representative kinase assay. Myelin basic protein (*MBP*) phosphorylation is shown in the *upper panel*, and MAPK levels (αHA) are shown in the *bottom panel*. *E*, the cooperative activation of Elk-1 by intersectin and EGF is blocked by U0126. Experiment was performed as in *C*. Data are expressed as the mean ± S.E. for three independent experiments performed in duplicate.

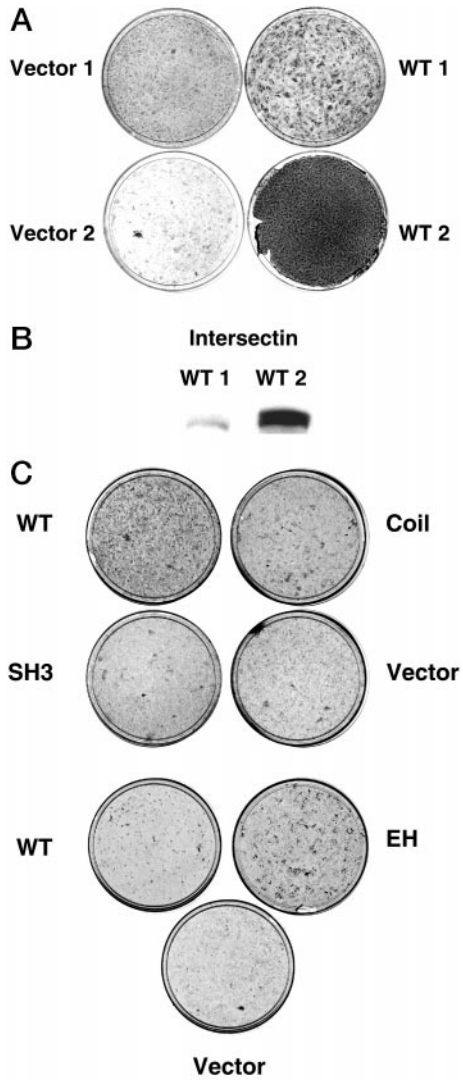

FIG. 3. **Intersectin expression induces transformation of NIH/3T3 cells.** *A*, NIH/3T3 cells were transfected with either vector alone or the intersectin expression construct and then selected in hygromycin. Clonal cell lines were isolated from each transfection and analyzed for levels of expression of intersectin by Western blot analysis. Clones with different levels of expression where then replated from cryogenic storage for a 2° focus formation assay as described under "Experimental Procedures." *B*, anti-HA Western blot of intersectin clonal cell lines showing relative differences in protein expression between individual clonal lines. Note that the relative differences in protein expression between the lines correlates with the relative degree of transformation seen in *A*. *C*, NIH/3T3 cells were transfected with the various intersectin expression constructs and polyclonal cell lines were isolated after selection in hygromycin as in *A*. These polyclonal lines were then analyzed for transformation in a 2° focus formation assay as described under "Experimental Procedures."

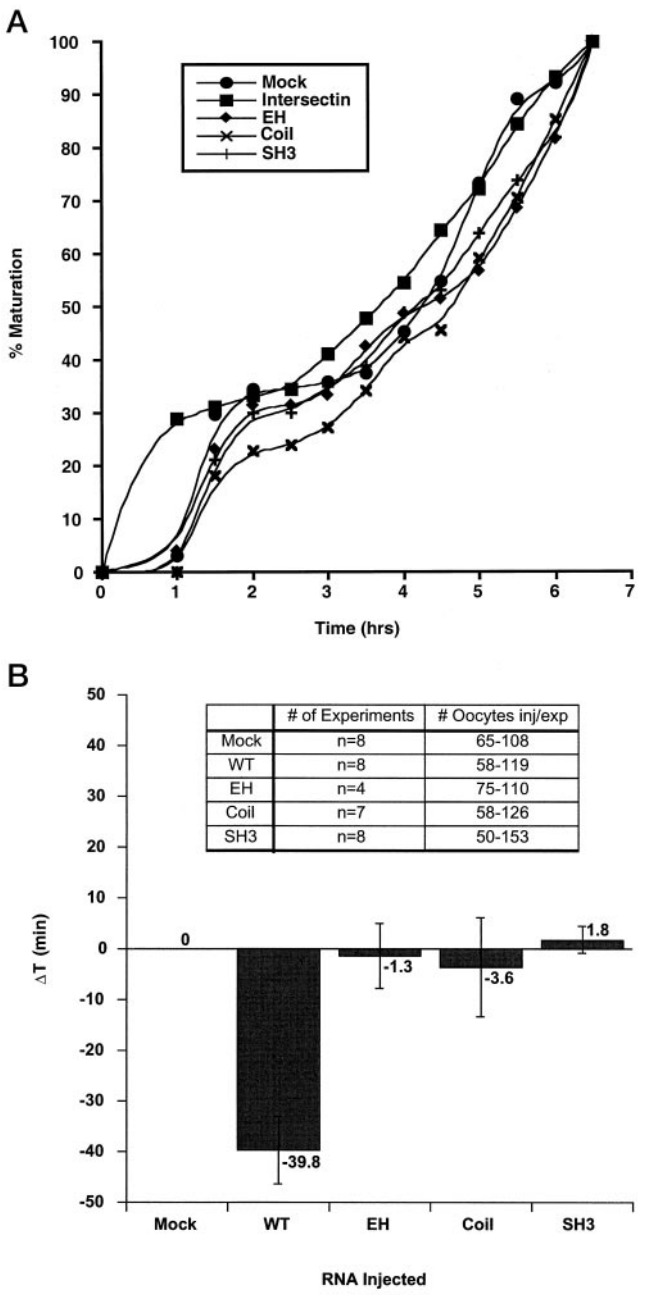

FIG. 4. **Expression of full-length intersectin accelerates progesterone-induced oocyte maturation.** *A*, results from a representative maturation experiment. Data are expressed as the percentage of oocytes that underwent GVBD by the indicated times (hours). Oocytes were injected with the following: ●, mock; ■, intersectin; ♦, EH; ×, coil; +, SH3. *B*, quantitation of acceleration in maturation rate. Data are expressed as the average difference in time for 50% of the oocytes to have undergone GVBD relative to buffer (mock) injected samples. The data in the table indicate the number of independent experiments performed for each construct along with the number of oocytes injected with each construct. *Error bars* represent mean ± S.E.

2*D*). Thus, these data suggest that intersectin may regulate EGFR activation of MAPK.

Intersectin Cooperates with Hormones to Accelerate Differentiation of Xenopus Oocytes—To further test whether intersectin possesses signaling activity, we expressed the various intersectin proteins in *X. laevis* oocytes and measured their ability to induce germinal vesicle breakdown (GVBD) in injected oocytes. Unlike activated versions of Ras, Raf, MEK, and MAPK, expression of intersectin alone was insufficient to induce maturation; however, in the presence of progesterone, intersectin expression led to an acceleration in oocyte maturation by approximately 40 ± 6.7 min (S.E.) (Fig. 4). Similar results have been found with activated Src, which by itself is incapable of inducing GVBD but cooperates with progesterone to accelerate maturation (26). Interestingly, expression of the intersectin truncation mutants had no significant effect on progesterone-induced maturation. Thus, intersectin cooperates with endogenous signal transduction pathways to accelerate maturation of oocytes, further supporting the notion that intersectin, like other proteins such as Src, Ras, and Raf, has the capacity to activate mitogenic signaling pathways in cells.

DISCUSSION

The potential link between endocytosis and mitogenic signaling has recently been the focus of much interest (27, 28).

Although endocytosis serves a regulatory role in the attenuation of receptor signaling, we present evidence that components of the endocytic machinery, *e.g.* intersectin, may also activate mitogenic signaling pathways. A variety of biochemical data indicate that intersectin is an important component in clathrin-mediated endocytosis. Expression of the NH_2-terminal SH3 domain of intersectin (SH3A) inhibits intermediate stages of this process (12). In addition, expression of full-length intersectin inhibits endocytosis of the transferrin receptor (9). These results are likely due to the recruitment of endocytic components through the interaction with the EH, coiled-coil, and SH3 domains of intersectin. Indeed, multiple endocytic proteins such as epsin, Eps-15, dynamin, the AP2 complex, and clathrin can be found associated with intersectin *in vivo* (6, 9–11). Although important for endocytosis, we now demonstrate that intersectin is also capable of activating signal transduction pathways (*i.e.* Elk-1), inducing oncogenic transformation, and potentiating differentiation by hormones. These findings suggest that intersectin plays a dual role in the cell and, thus, provides a link between regulation of endocytosis and mitogenic signaling pathways.

Using transient transcriptional reporter assays as well as *in vitro* kinase assays, we demonstrated that intersectin expression activates the Elk-1 transcription factor in a p42/p44 Erk-independent manner. This finding is supported by the fact that a pharmacological inhibitor of MEK-1/2 (U0126) did not block Elk-1 activation by intersectin. In addition, we did not detect activation of Erk-2/MAPK in intersectin-expressing cells nor did we detect any enhancement in the activation of Erk-2/MAPK by EGF in these cells. In contrast, intersectin activated Elk-1 to levels 2- to 3-fold higher than the level obtained with maximal EGF stimulation (see Fig. 2). Although the related MAPK proteins p38 and Jnk have been shown to activate Elk-1 in some cell types (29), we have not been able to demonstrate an involvement of these kinases in the activation of Elk-1 by intersectin.[2] These results suggest that intersectin activates Elk-1 through a nonclassical MAPK pathway. Future work will be aimed at delineating this pathway.

The finding that the EH domains are the region of intersectin that activates Elk-1 suggests that targets of the EH domains are components in the pathway leading to Elk-1 activation. Recently, mouse epsin-1 and -2 were identified as ligands for the EH domains of Eps15 and intersectin (6, 22). Epsin is thought to function in clathrin-mediated endocytosis, because mutant forms of this protein as well as antibodies specific for epsin disrupt endocytosis (22). Furthermore, mutations in yeast epsins disrupt endocytosis (30). Based on these observations, we tested whether the *Xenopus* homolog of epsin, MP90, might be involved in the activation of Elk-1. Indeed, the *Drosophila* homolog of epsin, liquid facets, has been shown to be important for the proper function of the Sevenless RTK suggesting a role for this protein in signal transduction (31). Interestingly, expression of MP90, a protein initially discovered due to its phosphorylation during mitosis (19), activated Elk-1 only slightly but did not alter Elk-1 activation by EGF. These findings lead to two conclusions: first, the ability of intersectin to activate transcriptional pathways is not shared by all components of the endocytic machinery; and second, intersectin must associate with additional cellular components through the NH_2-terminal EH region in order to activate Elk-1. Although this association is likely through interaction with either of the individual EH domains alone or in combination, it is formally possible that the 109 amino acids between the EH domains may direct association. Expression of this region as well as the individual EH domains will help address this issue. Furthermore, these results do not rule out the possibility that MP90 is a component of the pathway leading to intersectin activation of Elk-1, but rather that MP90 expression alone is not sufficient for activation of Elk-1. The recent identification of the transcription factor promyelocytic leukemia zinc finger protein as an epsin binding protein (32) suggests that epsin may indeed be important in intersectin's signaling activity. Furthermore, the finding that epsin accumulates in the nucleus following Leptomycin B treatment further supports a role for this protein in nuclear events (32).

In addition to activating transient signaling pathways, intersectin expression led to oncogenic transformation of rodent fibroblasts. Although intersectin expression was sufficient to induce transformation, the transforming ability of intersectin was weak in comparison to an activated allele of H-Ras (H-Ras61L; data not shown). These findings are reminiscent of results obtained with the Eps15 protein. This EH family member was first identified as a substrate of the EGFR and was also shown to transform rodent fibroblasts (15). Interestingly, the gene encoding Eps15 has undergone rearrangement in certain leukemias (16). Although the importance of this rearrangement in the genesis of the disease is unclear, the finding that Eps15 expression is capable of inducing transformation suggests that this rearrangement, like the BCR-ABL translocation, may also play a role in the malignant progression of the disease. Similarly, the finding that intersectin induces transformation suggests that it too plays a role in human malignancies.

In this study we provide several lines of evidence that suggest intersectin cooperates with RTK signaling pathways. First, stimulation of intersectin-expressing cells with EGF led to a synergistic enhancement in Elk-1 activation. Although intersectin alone did not activate Erk-MAPK, the cooperativity between EGFR and intersectin with regard to Elk-1 activation was dependent on Erk, because treatment of cells with U0126 abolished the effect. This result further supports the notion that intersectin activates an Erk-independent pathway that, in combination with the EGFR-activated Erk pathway, synergistically activates Elk-1. Second, the activation of p42-Erk-2 by EGFR was partially inhibited by the SH3 domains of intersectin. Third, transformation by the EGFR was partially inhibited by the SH3 domains of intersectin.[4] The inhibition of both EGFR-induced MAPK activation and transformation by the SH3 domains of intersectin appears to be specific, because both oncogenic Src and Alk (33, 34) were unaffected by this intersectin mutant protein.[5] We believe this inhibition is due in part to inhibition of EGFR endocytosis by the SH3 domains of intersectin, because these domains block endocytosis (12). Indeed, work from McPherson and colleagues suggests that the SH3 domains of intersectin are sufficient to inhibit endocytosis of the EGFR.[6] However, recent work from McPherson and colleagues demonstrates that intersectin forms a complex with the Ras exchange factor Sos (14). Furthermore, they demonstrate that expression of the SH3 domains of intersectin is sufficient to block EGF activation of MAPK through inhibition of Ras activation.[7] Together, these data suggest that intersectin regulates MAPK activation through regulation of EGFR endocytosis as well as Ras activation. Finally, coexpression of intersectin with the EGFR led to an enhancement in focus formation. Although this effect was subtle, there was a distinct difference in the appearance of foci in the intersectin-coex-

[4] A. Adams and J. P. O'Bryan, unpublished observations.
[5] R. Mohney and J. P. O'Bryan, unpublished observations.
[6] P. S. McPherson, personal communication.
[7] Tong, X.-K., Hussain, N. K., Adams, A., O'Bryan, J. P., and McPherson, P. S. (July 13, 2000) *J. Biol. Chem.* 10.1074/jbc.M004096200.

pressing cells as compared with vector-transfected cells. Furthermore, similar results were obtained in clonal cell lines expressing the various intersectin constructs (data not shown). Collectively, these data suggest that intersectin has the capacity to influence certain RTKs (*i.e.* EGFR) and serves as a potential link between these receptors and the endocytic machinery. This notion is further supported by the isolation of Dap160, the *Drosophila* homolog of intersectin, in a genetic screen for modifiers of the Sevenless receptor tyrosine kinase.[8]

Although we demonstrate that intersectin potently activates Elk-1, it is possible that intersectin may activate other transcriptional pathways in addition to Elk-1. Due to the high level of constitutive activation of a number of reporter constructs in the 293T system, we have not been able to test this possibility. Using additional cell lines, we have begun to address whether intersectin may regulate transcriptional pathways in addition to the Elk-1 pathway. These experiments may provide clues as to the protein(s) linking intersectin with stimulation of Elk-1 activity.

The notion that intersectin activates signal transduction pathways is further supported by the results of the *Xenopus* oocyte maturation experiments. Numerous oncoproteins such as Ras, Met, Trk, and Src (26, 35–38) have been shown to induce maturation of oocytes. The ability of these proteins to induce maturation correlates with their ability to induce signal transduction pathways. In the case of Ras, constitutive activation of this protein is sufficient to induce maturation in the absence of added hormone (35). However, expression of activated Src does not induce maturation on its own but rather accelerates progesterone-induced maturation (26). Similarly, intersectin is capable of accelerating progesterone-induced maturation of *Xenopus* oocytes implying that, like activated Src, intersectin is also capable of stimulating signaling pathways that cooperate with progesterone to accelerate maturation.

Intersectin expression is detected in most cell types with a larger mRNA present in brain-derived samples. Recent cloning of both human and mouse intersectin homologs indicates that the larger isoform arises from differential splicing at the 3′-portion of the gene resulting in the addition of DH, PH, and C2 domains at the COOH terminus (7). Given the homology of the DH/PH domains of intersectin to other Dbl family members that regulate Rho GTPases and the involvement of Rho family proteins in endocytosis (39), this larger version of intersectin may also function to regulate Rho activity and couple Rho to sites of clathrin-mediated endocytosis.

The ability of intersectin to cooperate with growth factor and hormone-regulated signaling pathways may involve interaction with other EH domain-containing proteins such as Eps15, a known substrate of the EGFR (15). EH-containing proteins are thought to function through heteromerization, which appears to be promoted in part by the coiled-coil domain. This region of Eps15 assembles into tetramers that consist of parallel dimers arranged in a larger dimeric, antiparallel structure (40). In addition, recent work on Ese1, the mouse homolog of intersectin, indicates that the coil domain can promote heteromerization between intersectin and Eps15 (9).[2] Thus, heteromeric interaction of certain EH domain family members may promote diverse protein complexes with distinct signaling activities.

The results presented here indicate that intersectin activates signaling pathways in addition to regulating endocytosis. The ability of intersectin to activate signal transduction pathways (*i.e.* Elk-1) may involve interaction with cellular ligands of its EH domains other than epsin. A variety of EH domain ligands that carry the Asp-Pro-Phe (NPF) motif have been described (6, 41), one of which may be responsible for Elk-1 activation. Future experiments will address the identification of the molecules mediating intersectin's effects on signal transduction processes.

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[8] F. Rintelen and E. Hafen, personal communication.

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Intersectin, an Adaptor Protein Involved in Clathrin-mediated Endocytosis, Activates Mitogenic Signaling Pathways

Anthony Adams, Judith M. Thorn, Montarop Yamabhai, Brian K. Kay and John P. O'Bryan

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