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Modulation of HeLa Cells Spreading by the Non-Receptor Tyrosine Kinase ACK-2

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Abstract The CDC42 regulated non-receptor tyrosine kinase ACK-2 has been associated with integrin signaling. In this report, the effect of ACK-2 on the modulation of cell spreading and motility was examined. HeLa cells expressing epitope-tagged wild type ACK-2 showed a slower rate of spreading on fibronectin when compared with untransfected cells. An ACK-2 protein lacking its SH3 domain was still capable of modulating HeLa cell spreading suggesting that its tyrosine kinase activity is sufficient to induce the observed phenotype. The ACK-2 effect on the rate of cell spreading did not involve inhibition of integrin-mediated activation of PI-3K signaling, since it did not alter membrane translocation of a GFP-PH-AKT domain (AKT pleckstrin homology domain) used as a reporter for PI-3K products induced by cell adhesion. The ACK-2 effect appears to be upstream from the adapter protein CrkII, since co-expression of CrkII and ACK-2 results in a neutralization of ACK-2 mediated effects on HeLa cell spreading. Similarly, co-expression of p130Cas, which interacts with the adapter protein CrkII, with ACK-2, also results in a partial reversion of the ACK-2 effects on cell spreading. CrkII mediated reversal of the ACK-2 induced phenotype requires the activity of the small GTPase, Rap1. Co-expression of ACK-2 and CrkII with a dominant negative form of Rap1 reverses the neutralization by CrkII suggesting that CrkII mediated activation of Rap1 is required. However, an active form of Rap1 is not sufficient to reverse the ACK-2 phenotype by itself. A role for Rac1 in ACK-2 effects was also established. An activated Rac1 protein neutralized the ACK-2 mediated inhibition of cell spreading. A direct measurement of cell motility by either a modified Boyden chamber or wounding assay demonstrates that ACK-2 overexpression increases the motility of the cells. These results suggest that ACK-2 modulates HeLa cells spreading upstream of pathways regulated by CrkII and that ACK-2 may regulate cell motility by controlling the activation of small GTPases such as Rap1 and Rac1. J. Cell. Biochem. 84: 655–665, 2002.

Key words: HeLa cells; integrin; tyrosine kinases; adhesion; GTPases

Cell motility is a crucial event involved in the immune response, wound healing, and morphogenesis [Schwartz, 1993; Springer, 1994; Matsumoto et al., 1995; Schwartz et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996; Gumbiner, 1996; Streit et al., 1996]. Adherent cells utilize extracellular matrix proteins to modulate cell movement via cell surface receptors that include integrins [Hynes, 1992]. The binding of ligands to integrins initiates a complex signaling cascade that involves cellular structures such as focal adhesions and the actin-myosin cytoskeleton [Clark and Brugge, 1995; O'Neill et al., 2000]. These events in turn modulate the signaling of growth factor receptors to control cell motility, growth, and differentiation [Hynes, 1992; Clark, 1994; Clark and Brugge, 1995; Hall, 1998; Sieg et al., 2000].

Among the pathways controlling the actin-based cytoskeleton is the signaling cascade initiated by the activated small GTP-binding proteins of the Rho-gene family, Rho, Rac, and CDC42 [Bar-Sagi and Hall, 2000; Bishop and Hall, 2000; Evers et al., 2000; Ridley, 2000; Symons and Settleman, 2000]. Ectopic expression of activated forms of these proteins induce remarkable changes in the actin cytoskeleton and modulate cell adhesion, cell spreading, and motility [Bishop and Hall, 2000; Evers et al., 2000; Ridley, 2000]. The array of cellular responses elicited by these proteins is likely due to interactions with downstream targets that include kinases and adapter proteins [Bar-Sagi

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and Hall, 2000; Bishop and Hall, 2000; Evers et al., 2000].

Protein phosphorylation has been demonstrated to be a crucial modulator of integrin signaling [Clark and Brugge, 1995; O'Neill et al., 2000]. As such, kinase activation in response to cell adhesion is a common point of regulation with growth factor mediated cell motility. Among known activators of kinases in response to cell adhesion are the Rho-like small GTPases. Although much is known about the nature and regulation of serine/threonine kinases by the Rho-like GTPases [Bar-Sagi and Hall, 2000; Bishop and Hall, 2000; Ridley, 2000], the regulation of tyrosine kinases by these GTPases is less well characterized. The ACK proteins are non-receptor tyrosine kinases that specifically interact with the CDC42 GTPase. Today, two forms of the ACK proteins have been described, ACK-1 and ACK-2 [Manser et al., 1993; Yang and Cerione, 1997]. ACK-1 is a 120-kDa protein containing a kinase domain, an SH3 domain, a CDC42 binding domain, and a proline rich carboxyl-terminal domain. ACK-2 has the same basic structural features of ACK-1, but it has a shorter C-terminus tail. ACK-1 has been shown to undergo tyrosine phosphorylation in response to stress signals such as temperature shift, hyperosmotic shock, and growth factor stimulation [Satoh et al., 1996] as well by removal of fibronectin matrix from HUVECs [Bourdoulous et al., 1998]. In addition, an interaction between ACK-1 and Grb2 has been described suggesting that this protein may participate in growth factor signaling [Satoh et al., 1996]. A physiological role for ACK-1 has been recently proposed as a regulator of GTP-exchange factors activity toward Rho, CDC42, and Ras by directly phosphorylating Dbl and Ras-GRF1 exchange factors [Kato et al., 2000; Kiyono et al., 2000]. ACK-2 has also been shown to be regulated by some growth factors [Yang and Cerione, 1997] and by integrin-mediated cell adhesion [Yang et al., 1999]. It is unknown whether ACK-2 also regulates the activity of exchange factors as ACK-1 does.

The described integrin-mediated activation of ACK-2 has prompted us to investigate whether ectopic expression of ACK-2 would result in modulation of the integrin-mediated motility of HeLa cells. In this article, we describe results that suggest that ACK-2 participates in the dynamic regulation of cell spreading by modifying cellular events regulated by the CrkII-Rap1 axis.

MATERIALS AND METHODS

Expression Vectors

ACK-2 cDNA [Yang and Cerione, 1997] was obtained by PCR using quick-clone bovine brain cDNA library (Clontech, Palo Alto, CA). The following primers were used for the PCR: 5′CAGCATGCAGCCAGAGGAGGGCAC and 3′TCACCCCCAGGGCTGGGTGAGG. The PCR product was then subcloned into pCMV-myc (Stratagene, La Jolla, CA), pHM6-HA (Roche Molecular Biochemicals, Indianapolis, IN), and pEGFP (Clontech). Site directed mutagenesis (Stratagene) using the primers 5′ CGTGGCTGTGAGGTGCCTGAAGCC and 3′ GGCTTCAGGCACCTCACAGCCACG constructed a kinase dead mutant (K158R). PCR with the primers, 5′ CAGCATGCAGCCAGAGGAGGGCAC and 3′ CAGGTTCTTCGAAGTCCTG produced a cDNA that encodes for an ACK-2 protein lacking its SH3 domain.

Rap1 cDNA was cloned from Human placenta cDNA (Clontech), using the PCR primers, 5′ATCATGCGTGAGTACAAGC and 3′CTAGAGCAGCAGACATG. An activated form of Rap1 (RapG12V) and a dominant negative form (Rap1S17N) were obtained by site directed mutagenesis (Stratagene) with the primers: V12: 5′GGTCCTTGGTTCAG***T***AGGCGTTGGG and 3′CCCAACGCCT***A***CTGAACCAAGGACC and N17: 5′GGAGGCGTTGGGAAG***AA***TGCTCTGACAG and 3′CTGTCAGAGCA***TT***CTTCCCAACGCCTCC. All RAP1 constructs were subcloned into a pSG5 vector (Stratagene) with an N-terminal GST tag, obtained from Scott Weiss at Pfizer.

ROCK active and K105A mutants were made as described [Itoh et al., 1999]. The kinase dead LIMK was prepared as described [Yang et al., 1998]. All the cDNAs were sequenced to confirm the correct sequence.

Additional expression vectors used in this study were kindly provided by the following sources: CDC42 N17, RacN17, RhoL63, and CDC42L61, were gifts from Alan Hall, RacV12 was from Onyx Pharmaceuticals (CA), CrkII and CrkA38 from Motti Anafi, p130Cas from Bruce Mayer and RhoN19 from Ian Macara.

Cell Culture and Transfection

HeLa cells were grown in DMEM F12 w/ GlutaMax plus 10% fetal bovine serum (FBS) at 37°C, 5% CO_2. Transfections were performed using LipofectAMINE PLUS according to the

manufacturer's protocol (Life Technologies, Rockville, MD). Protein expression was confirmed by immunoblotting cell lysates with antibodies directed to the specific tag used (HA: HAF7; c-myc: 9E10 both obtained from Santa Cruz Biotechnology, Santa Cruz, CA). MDCK cells were grown in DMEM F12 plus 10% FBS at 37°C, 5% CO_2. Transfections were performed using LipofectAMINE 2000 according to the manufacturer's protocol (Life Technologies).

Spreading Assay

HeLa cells in six well plates were transfected with 1.5–2 μg of DNA. Twenty-four hours post transfection the cells were trypsinized, washed twice with DMEM F12 w/GlutaMax supplemented with 0.2% bovine serum albumin, resuspended in DMEM F12 w/GlutaMax plus 0.5% FBS, and allowed to recover at 37°C, 5% CO_2 for 30 min. A portion of the cells were plated on LAB-TEK four chamber slides (Nunc, Naperville, IL) that were coated with fibronectin (50 μg/ml) and incubated for 30 min at 37°C, 5% CO_2. The cells were washed with Dulbecco's PBS, fixed with 10% formalin for 20 min (Stephens Scientific, Riverdale, NJ) and processed for immunostaining with specific antibodies as previously described [Herrera, 1998]. Texas red or Oregon green coupled antibodies were used as secondary antibodies as indicated in Figures 2C and 3C (Molecular Probes, Eugene, OR). TRITC labeled Phalloidin (Sigma, St. Louis, MO) was used for F-actin staining. Cells expressing the specific proteins were counted and their spreading, estimated by actin labeling, was calculated as a percentage of the total number of cells counted (N = 100–300). The results are expressed as percentage of spreading observed in control cells.

Immunoprecipitations and Immunoblots

For the analysis of protein expression either by immunoprecipitation or immunoblot, cells were harvested in HY buffer (50 mM HEPES, pH 7.5, 1% Triton X-100, 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 M NaF, 0.01 M sodium pyrophosphate, 10% glycerol, 3 mM sodium orthovanadate, 50 mM β-glycerophosphate), protease inhibitors (10 μg/ml leupeptin and 10 μg/ml aprotinin) and processed as previously described [Petruzzelli et al., 1996] with the exception that the immune complex was adsorbed using Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology). The anti-HA mouse monoclonal 12CA5 antibody (Roche Molecular Biochemicals) was used during the immunoprecipitation. For probing with an anti-phosphotyrosine antibody, a mix of mouse anti pTyr-4G10 (Upstate Biotechnology, Lake Placid, NY) and mouse anti pTyr-PY20 (Transduction Labs, Lexington, KY) were used. Detection of the immune complex was carried out by ECL (Amersham).

Wounding Assay

HeLa cells plated in 100-mm dishes were transfected with 9 μg DNA of vector expressing GFP or GFP-ACK-2. After 24 h, a wound was created with a sterile razor blade in each of the plates. The number of GFP expressing cells was determined, and the percent migration was determined as the percent GFP expressing cells present in the wound area divided by the percent of total cells present in the same area.

Modified Boyden Chambers Assay

HeLa cells were transfected with vectors expressing GFP or GFP-ACK-2 and harvested as described in the spreading assay; 13,000 GFP expressing cells per trans-well were loaded in the top of the filter that had been pre-coated with 25 μg/ml fibronectin in the bottom. Media (600 μl) were added to the lower chamber. The cells were incubated for 5 h at 37°C and 5% CO_2. The filters were cut from the well and mounted on a slide for analysis. The number of GFP positive cells was determined under fluorensce microscopy.

RESULTS

Expression of Epitope Tagged ACK-2

The cDNA encoding for wild type kinase or SH3 domain deficient ACK-2 (Fig. 1A) protein was tagged with HA or c-myc epitopes and analyzed for expression and tyrosine autophosphorylation in HeLa cells. All forms were expressed when probed with the specific antibody. As shown in Figure 1B, the wild type and the SH3 deletion, but not the kinase deficient ACK-2 protein, are autophosphorylated on tyrosine. In addition, the wild type ACK-2 is associated with kinase activity toward exogenous substrate.

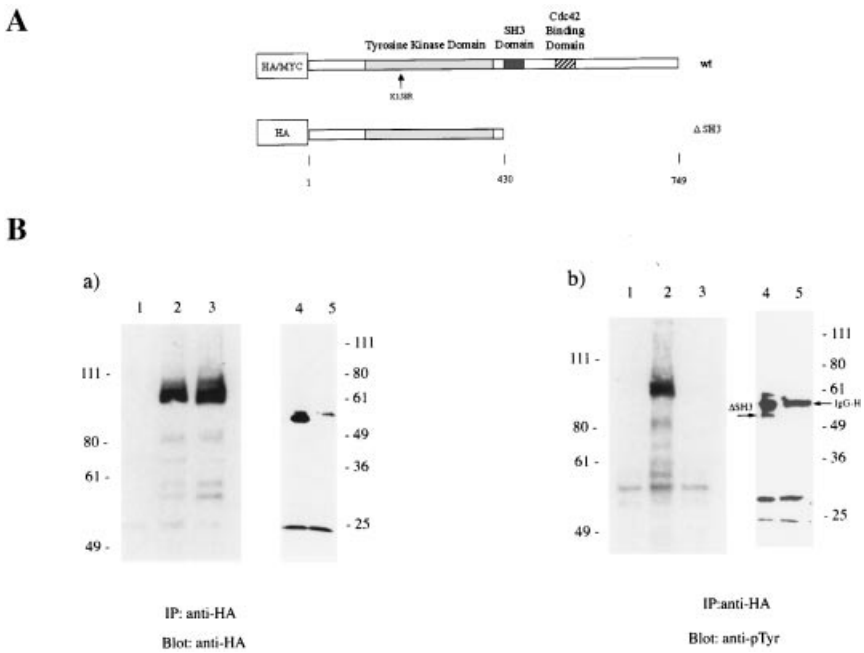


Fig. 1. Expression and tyrosine phosphorylation of ACK-2 proteins. **A**: Schematic representation of ACK-2 depicting its functional domains and the position of the epitope tags used in the construction of the expression vector as described in Materials and Methods. **B**: The expression and tyrosine phosphorylation status of the wild type (**lane 2**), kinase deficient (**lane 3**), or SH3 deleted (**lane 4**). HA-tagged ACK-2 in transfected HeLa cells is shown by anti-HA blot (**a**: lanes 1–5) or anti-phosphotyrosine blot (**b**: lanes 1–5). Cells transfected with the empty vector are shown in **lanes 1** and **5**.

ACK-2 Regulates Cell Spreading

ACK-2 has been shown to be activated via integrin receptors ligation with substrata [Yang et al., 1999]. Therefore, we studied the effect of ACK-2 expression in an integrin dependent process such as cell spreading. We observed that the spreading of HeLa cells on fibronectin was significantly modified by the expression of ACK-2 (Fig. 2A). Cells expressing wild type or the SH3 deleted ACK-2 showed diminished spreading as compared with untransfected or vector alone transfected cells (Fig. 2B). An ACK-2 protein that contains a mutation that renders it kinase deficient showed a reduced effect suggesting that the ACK-2 mediated phenotype is dependent, in part, on its kinase activity. A time course analysis showed that cells expressing ACK-2 reached the full spread phenotype at a slower rate (Fig. 2C), suggesting the effect of ACK-2 on HeLa cells spreading is in the modulation of the rate of spreading. We have made a GFP-ACK-2 fusion protein in order to study the subcellular localization of the protein during a dynamic assay such as cell spreading or static assay (monolayer culture). As shown in Figure 3A, the GFP-ACK-2 protein retains the ability to undergo tyrosine autophosphorylation and mimics the effect of wild type ACK-2 on HeLa cell spreading (Fig. 3B). HeLa cells cultured and transfected in a monolayer show a unique pattern of expression with the distinct formation of concentrated pockets of protein expression as detected by direct immunofluorescence (Fig. 3C). This pattern is partially modified when HeLa cells are spread; some movement of the GFP-ACK-2 fusion protein toward the center and boundaries of the cell is seen.

ACK-2 Effects Are Modulated by CrkII, p130Cas, and Rap1

The mechanism by which ACK-2 regulates the rate of cell spreading is not known. In an attempt to identify signaling pathways that are involved in the ACK-2 mediated alteration of cell spreading, we have analyzed the role of proteins previously described to be involved in the control of integrin signaling. First, we studied whether the ACK-2 induced phenotype was mediated by altering the integrin-mediated activation of PI-3K. To study this, we used a GFP-PH-AKT as a reporter for the activity of PI-3K [Raucher et al., 2000]. We studied the membrane translocation of this fusion protein in response to integrin mediated adhesion in

cells upon co-transfection with ACK-2. As shown in Figure 4, the presence of ACK-2 did not block the ability of the GFP-PH-AKT protein to concentrate at the cell membrane. This indicates that PI-3K activation in response to integrin signaling is intact and suggests that ACK-2 effects on cell spreading are either downstream or in a parallel pathway to PI-3K activity.

The role that small GTPases play in ACK-2 mediated modulation of cell spreading was then studied. As shown in Figure 5A, the effect of ACK-2 was not neutralized by co-transfection with dominant negative forms of Rac or Rap1. It was partially neutralized by dominant negative form of Rho and strongly neutralized by negative form of CDC42. Dominant negative forms of downstream effectors of both Rho and Rac such as LIMK or ROCK were also weak neutralizing agents of the ACK-2 effects on cell spreading (Fig. 5A). On the other hand, co-transfection of ACK-2 with activated forms of these signaling molecules demonstrated that the effect of ACK-2 on the rate of cell spreading could be modulated or neutralized by the activated form of Rac1, but not by activated Rho, Rap1, CDC42 or its downstream target, ROCK (Fig. 5B). We also analyzed the role of CrkII, an adapter protein containing both SH3 and SH2 domains [Reichman et al., 1992], in ACK-2 modulation of cell spreading. As shown in Figure 6, co-transfection of ACK-2 with CrkII results in a neutralization of ACK-2 effects on cell spreading. Interestingly the effect of CrkII was impaired by inactivation of its SH2 domain suggesting that this may be mediated by its ability to bind to tyrosine phosphorylated proteins such as p130Cas. Accordingly, co-transfection of ACK-2 with a GST-p130Cas, a partner for the SH2 domain of CrkII, also resulted in neutralization of ACK-2 effects. This observation suggests that p130Cas may be involved in this process by its association with the SH2 domain of the endogenous CrkII protein. The effect of CrkII on the ACK-2 induced phenotype required the activity of Rap1 since co-transfection of CrkII along with ACK-2 and a dominant negative form of Rap1, Rap1N17 resulted in a blunted action of CrkII.

ACK-2 Expressing HeLa Cells Have Increased Motility

The effect of ACK-2 on spreading HeLa cells suggested that this protein might influence their motility. A direct measurement of cell motility was carried out using a trans-well as well as a wounding assay to estimate the effect of ACK-2 on HeLa cells. As shown in Figure 7, the expression of a GFP-ACK-2 fusion protein increases the migration of cells through the filter (random motility) as well as migration of cells toward the wounded area (directional motility).

DISCUSSION

The loss of regulation of cell motility is likely to play a crucial role in the development of cellular metastasis. The ability of tumor cells to metastasize is dependent on several factors

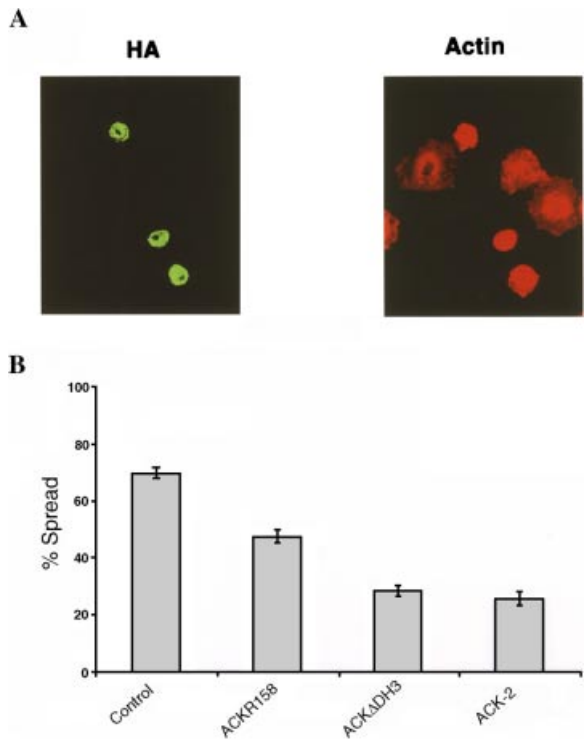


Fig. 2. ACK-2 expression alters HeLa cells spreading. **A**: HeLa cells that had been transiently transfected with HA-ACK-2 were allowed to undergo spreading as described in Materials and Methods. After fixation, cells were stained with anti-HA (green) antibodies and TRTC-phalloidin (red) and analyzed as described in Materials and Methods. **B**: Quantitative spreading assay of HeLa cells expressing wild type, kinase dead, or SH3 domain deleted forms of ACK-2. Assay and quantification was carried out as described in Materials and Methods. **C**: Time course of cells spreading. HeLa cells transiently transfected with vector alone (control) or wild type HA-ACK-2 (ACK-2) were allowed to spread on fibronectin for the indicated time points and processed and analyzed as described in Materials and Methods. Double stained (for ACK-2 and actin) cells were analyzed .The pictures depict the actin staining of the dually stained cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

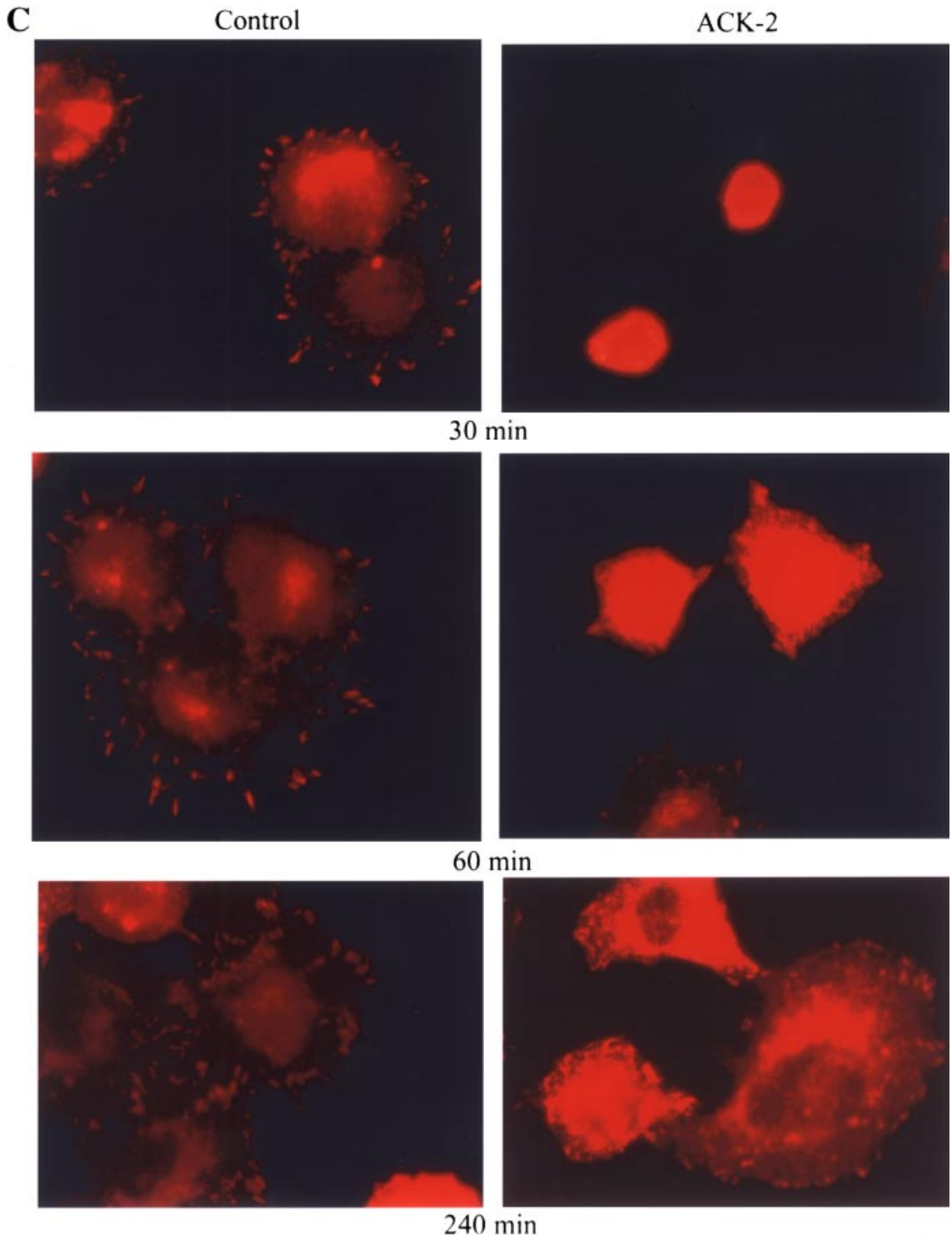


Fig. 2. (*Continued*)

that regulate cell–cell and cell–extracellular interactions [Schwartz, 1993; Agrez and Bates, 1994; Streit et al., 1996]. The biochemical signals involved in the control of cell motility must converge at the level of adhesion/de-adhesion, mediated by integrin receptors, and organization of the actin-myosin cytoskeleton [Clark and Brugge, 1995; Machesky and Insall, 1999]. Several pathways have been postulated to be involved in the control of cell motility that includes Ras/MAPK [Klemke et al., 1997; Herrera, 1998], PI-3K [Rickert et al., 2000],

PLC-g [Gual et al., 2000], Ena/VASP [Machesky, 2000], and the WASP/Arp2/3 [Machesky and Insall, 1999] pathways.

The Rho-like family of GTPases plays an important regulatory role in the integration of signaling involved in the control of cell motility [Bar-Sagi and Hall, 2000; Bishop and Hall, 2000; Evers et al., 2000; Ridley, 2000]. Several down-stream effectors of the Rho family of GTPases that participate in the assembly/disassembly of actin-based cytoskeleton have been described [Bishop and Hall, 2000; Evers et al., 2000]. Here we have studied the role that ACK-2, a target of activated CDC42, plays in the process of cell spreading.

Transient ectopic expression of GFP-ACK-2 or epitope-tagged ACK-2 in attached HeLa cells does not induce an obvious morphological phenotype (Fig. 3A, and data not shown). However, when HeLa cells expressing either epitope-tagged or GFP-ACK-2 fusion proteins are allowed to undergo spreading on fibronectin, the rate to reach the spread state is much slower than that seen in control cells (Figs. 2C and 3B). This indicates that ACK-2 modulates the rate of cell spreading and links the integrin-mediated activation of ACK-2 [Yang and Cerione, 1997] to the regulation of cell motility. The modulation of cell spreading by ACK-2 appears to require the ability of the protein to undergo tyrosine phosphorylation, since a kinase-deficient form of ACK-2 was not active in the spreading assay (Fig. 2B). The cellular substrates for ACK-2 are not known and it remains to be determined whether the Ras, Rho, and Rac exchange factors that have been described to be substrates for ACK-1 [Kato et al., 2000; Kiyono et al., 2000] are also targets for ACK-2. However, a casual relationship between melanoma cells spreading and ACK-1 activation has been described [Eisenmann et al., 1999] suggesting that these proteins may play a role in the aberrant motility associated with tumor formation [Schwartz, 1993; Agrez and Bates, 1994]. Noteworthy is the fact that ACK-1 and ACK-2 share high homology with the family of focal adhesion kinases, FAK and Pky2 [Yang and Cerione, 1997]. It is well known that FAK plays a crucial role in the regulation of cell motility [Sieg et al., 2000].

The molecular mechanism by which ACK-2 modifies rate of cell spreading is not known. However, we have established that ectopic expression of ACK-2 induces changes in the rate of cell spreading without interfering with the integrin-mediated PI-3K activation. Co-transfection of GFP-PH-AKT with ACK-2 did not result in impaired translocation of the GFP-AKT/PH fusion protein toward the boundaries of the cell in a spreading assay (Fig. 4). As

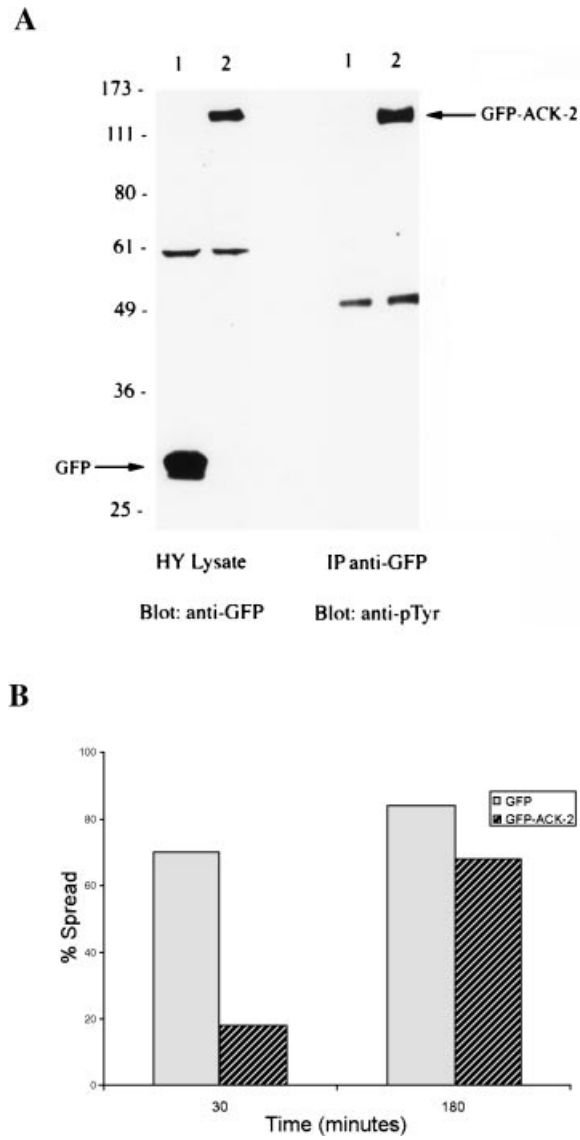
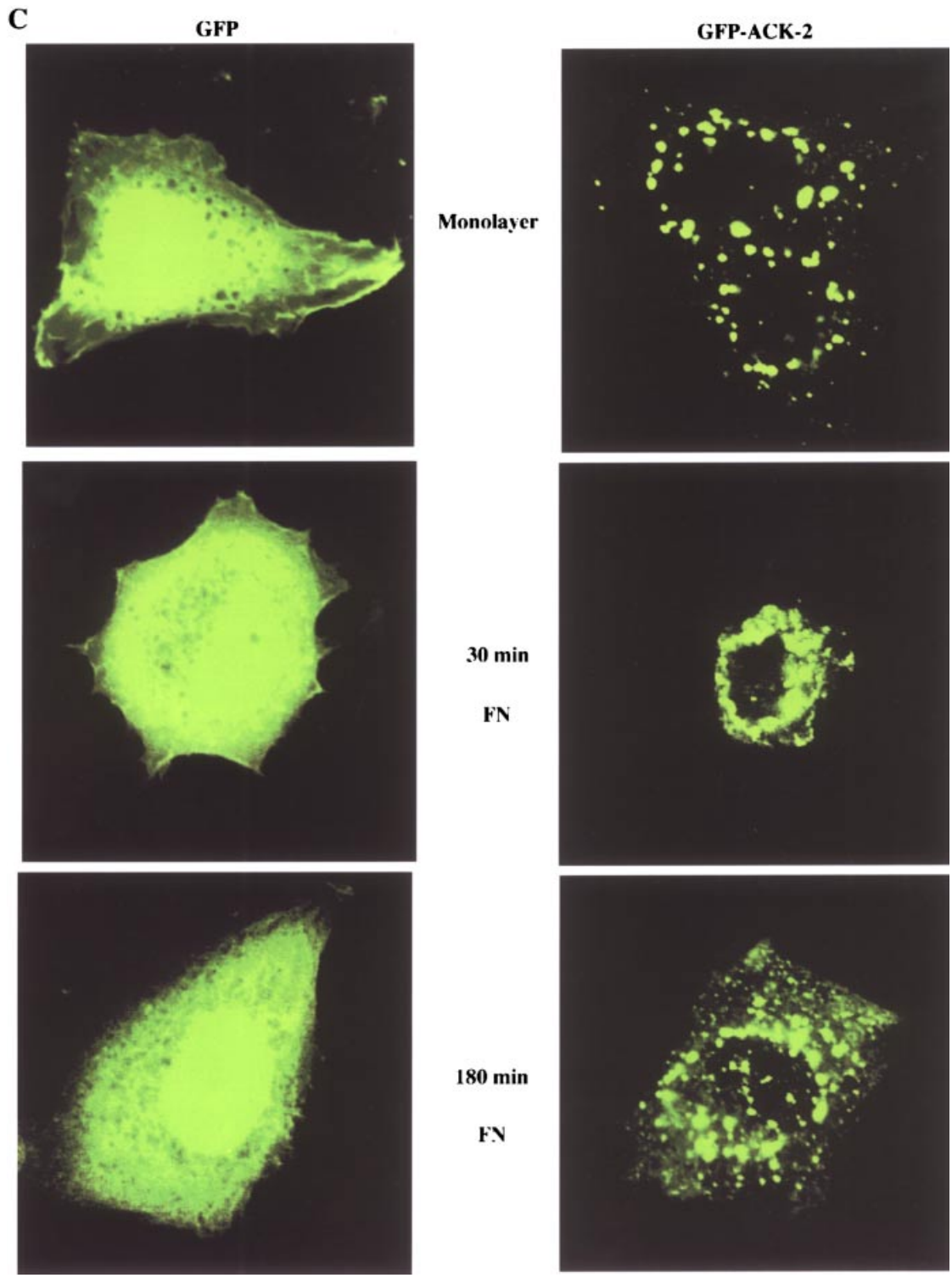


Fig. 3. GFP-ACK-2 expression and subcellular localization. **A**: Cell lysates were prepared and analyzed for the presence of GFP-ACK-2 by immunoblot using anti-GFP or anti-phosphotyrosine antibodies as described in Materials and Methods. **B**: Spreading assay for the GFP-ACK-2 fusion proteins was carried out as described in Materials and Methods. **C**: HeLa cells were transfected with GFP (**lane 1**) or GFP-ACK-2 (**lane 2**) containing vectors and fixed before (monolayer) or after spreading (FN) and analyzed by direct immunofluorescence using a confocal microscope as described in Materials and Methods. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]



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expected, a dominant negative form of CDC42 neutralized the ACK-2 effects on cell spreading, likely by interfering with endogenous CDC42-mediated activation of ACK-2. The activated form of CDC42 did not enhance the strong phenotype induced by ACK-2. We interpreted these results that the pool size of endogenous activated CDC42 generated by integrin-

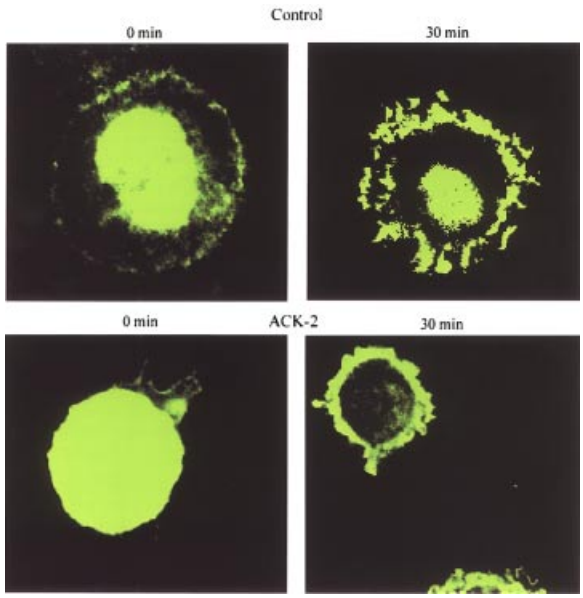

Fig. 4. Modulation of cell spreading by ACK-2 does not alter PI-3K activity. HeLa cells were co-transfected with GFP-PH-AKT with or without ACK-2 and allowed to spread on FN. After 30 min, cells were fixed and the GFP-PH-AKT protein was visualized by direct immunofluorescence using confocal microscopy. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

mediated adhesion is sufficient to activate the transfected ACK-2. Additional exploration of known signaling pathways involved in the control of actin-myosin cytoskeleton revealed that ACK-2 effects could be neutralized by an activated form of the small GTPase Rac1, suggesting that the overall effect of ACK-2 expression is to interfere with the integrin-mediated activation of the above proteins. In agreement with this hypothesis, the ACK-2 effects could also be neutralized by co-transfection of c-CrkII, an adapter involved in integrin signaling [Buensuceso and O'Toole, 2000; Cho and Klemke, 2000; Petruzzelli et al., 1996; Uemura and Griffin, 1999]. Given the observation that the SH2 domain of c-CrkII was required for the neutralization of ACK-2 effects, it is likely that its association with tyrosine phosphorylated proteins such as p130Cas is involved. It remains to be determined whether the role of c-CrkII in this process also involves the participation of its two SH3 domains. We also observed a partial neutralization of ACK-2 effects by over-expressing a GST-p130Cas protein. It is also a possibility that p130Cas may be involved in this process by its association with the SH2 domain of the endogenous CrkII protein. It has previously been established that

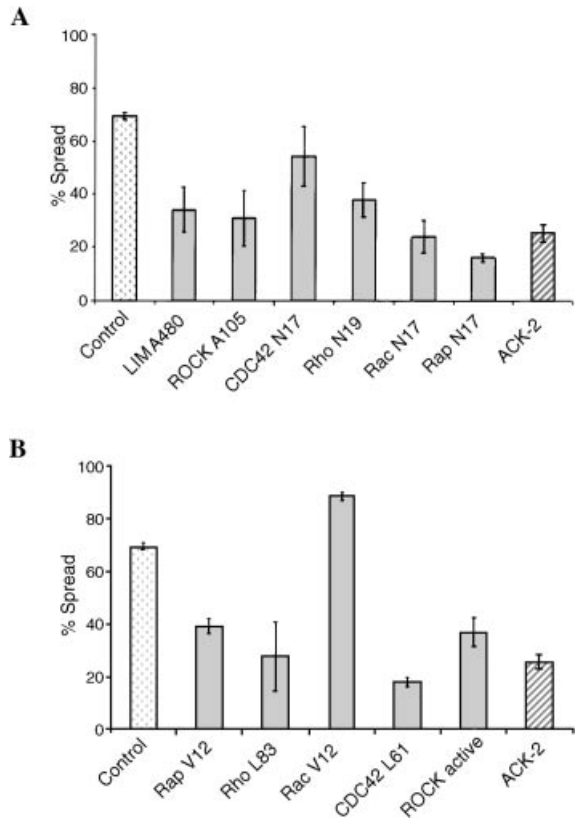

Fig. 5. Signaling molecules modulating ACK-2 effects on cell spreading. HeLa cells were co-transfected with ACK-2 and vectors expressing the indicated proteins. The spreading assay was carried out as described in Materials and Methods. **A**: Modification of ACK-2 effects by dominant negative forms of the following proteins: LIMK, ROCK, CDC42, Rho, Rac, and Rap1. **B**: Modification of ACK-2 effects by activated forms of the above-described proteins.

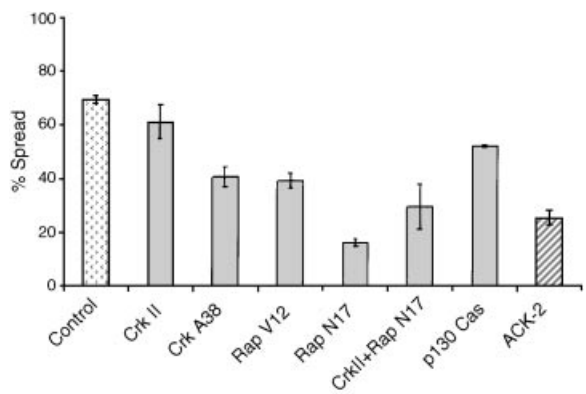

Fig. 6. CrkII neutralization of ACK-2 action during cell spreading. HeLa cells were co-transfected with ACK-2 and a vector expressing the wild type c-Crk, a SH2 domain mutant c-Crk, Rap1, or p130GST-Cas. Control depicts cells transfected with empty vector, and ACK-2 depicts cells transfected with ACK-2 encoding vector alone. The proportion of spreading in response to expression of each of these proteins, was assayed as described in Materials and Methods.

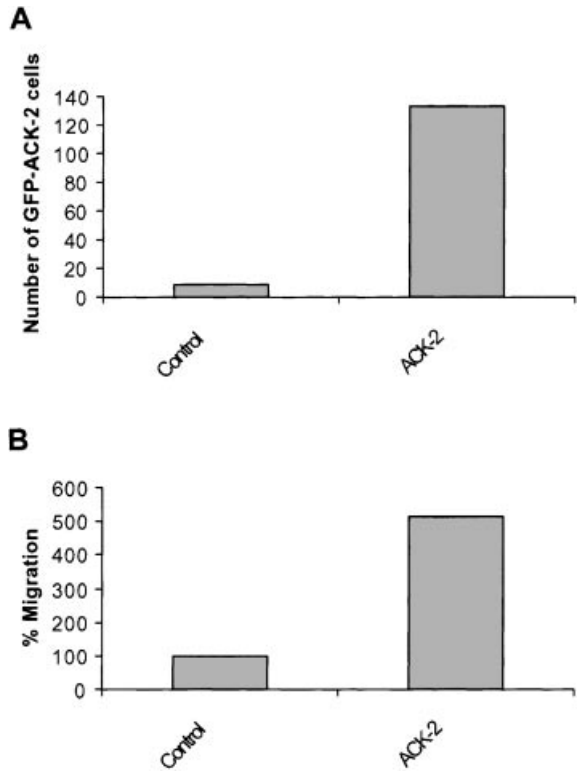

Fig. 7. ACK-2 increases HeLa cells migration. **A**: Trans-well migration assay. Cells were transfected with GFP or GFP-ACK-2 vectors as indicated in Materials and Methods. Equal number of GFP positive cells were allowed to migrate through the filter, and the cell number present after the 5 h was determined as indicated in Materials and Methods. **B**: Wounding assay. Cells were transfected with GFP or GFP-ACK-2 vectors as indicated in Materials and Methods. Cells were allowed to migrate to a wounded area of the dish and the percentage of green cells present in the wounded area was determined as indicated in Materials and Methods. A value of 100% was given to the cells expressing the GFP control.

p130Cas/CrkII coupling regulates cell migration [Klemke et al., 1998].

The effect of CrkII on the ACK-2 induced phenotype required the activity of Rap1, since co-transfection of CrkII along with ACK-2 and a dominant negative form of Rap1, Rap1N17, resulted in a blunted action of CrkII. However, activated Rap1 was not sufficient to mimic CrkII effects, suggesting that additional CrkII-dependent pathways are involved. In a simplistic interpretation of our data, it appears that ACK-2 effects may be due to prevention of the integrin-mediated activation of Rac1 and Rap1. Both activated Rac1 or over-expression of CrkII reversed the ACK-2 phenotype. At this moment, we do not know whether Rac1 lies upstream of CrkII, downstream, or in a parallel pathway. The action of CrkII requires the activation of Rap1, perhaps by its interaction with C3G, a known Rap1 exchange factor [Ichiba et al., 1999]. However, it appears that activated Rap1 is not sufficient to rescue the spreading of the ACK-2 expressing cells suggesting the involvement of additional, CrkII-regulated pathways.

The above-described observations suggest that ACK-2 may participate in the overall control of cell motility. In order to determine this directly, we measured HeLa cells motility under both directional and random motility. Our results (Fig. 7) support the conclusion that the mobile properties of the HeLa cells are enhanced by the expression of ACK-2. The molecular mechanism involved in ACK-2 enhanced cell motility is unknown. Recently, it has been demonstrated that ACK proteins bind to clathrin [Teo et al., 2001; Yang et al., 2001], thus it is possible that it modulates the integrins' residence time at the plasma membrane by enhancing their rate of internalization. This possibility is currently under investigation.

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