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# Ash/Grb-2, a SH2/SH3-containing protein, couples to signaling for mitogenesis and cytoskeletal reorganization by EGF and PDGF

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**The Src homology (SH) region 2 binds to phosphorylated tyrosine residues and SH3 domains may interact with cytoskeletal molecules and GTPase-activating proteins for Rho/Rac proteins (the small GTP-binding proteins related to Ras). The recently cloned Ash/Grb-2 protein, a 25–28 kDa molecule composed entirely of SH2 and SH3 domains, is a mammalian homolog of the *Caenorhabditis elegans* Sem-5 protein, which communicates between a receptor protein tyrosine kinase and a Ras protein. In the present study the function of Ash/Grb-2 was investigated by microinjecting cells with an anti-Ash antibody. The antibody abolished both S phase entry and the reorganization of actin assembly to ruffle formation upon stimulation with epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). On the other hand, anti-Ash antibody had no effect on S phase entry or actin stress fiber formation induced by either serum or lysophosphatidic acid. Since the induction of DNA synthesis, ruffle induction and stress fiber formation involve a function of Ras, Rac activation and Rho activation respectively, the findings strongly suggest that Ash plays a critical role in the signaling of both pathways downstream from growth factor receptors to Ras and Rac. Consistent with this, Ash co-precipitated with EGF receptor from EGF-stimulated cells. Other proteins of approximately 21, 29, 135 and 160 kDa were also detected in the anti-Ash antibody immunoprecipitates, suggesting a role of Ash as a linker molecule in signal transduction downstream of growth factor receptors.**

*Key words:* Ash/Grb-2/growth factor/Rac/Ras/Src homology region

## Introduction

Recently, Ash/Grb-2, a 25–28 kDa protein lacking any domains other than Src homology (SH) regions 2 and 3 in the order SH3-SH2-SH3, has been cloned (Lowenstein *et al.*, 1992; Matuoka *et al.*, 1992). This molecule is completely conserved between humans and rats and has been found to be the mammalian homolog of the *Caenorhabditis elegans* gene product, Sem-5. Sem-5 is involved in vulval induction, sex myoblast migration and larval survival and has been demonstrated to play a role between the receptor protein tyrosine kinase (PTK), Let-23, and the Ras protein, Let-60 (Clerk *et al.*, 1992). In addition, a *Drosophila* homolog of Sem-5 has been suggested to function between Sevenless (a receptor PTK) and ras 1 (Williams, 1992). By analogy, Ash is a potential signaling molecule between the receptor PTKs and Ras in mammalian cells. In fact, Ash binds to the receptors for epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) upon stimulation (Lowenstein *et al.*, 1992; Rozakis-Adcock *et al.*, 1992) and may play a role in mitogenesis (Lowenstein *et al.*, 1992; Matuoka *et al.*, 1992) and neurite outgrowth (Rozakis-Adcock *et al.*, 1992), both Ras-dependent functions (Bourne *et al.*, 1990; Hall, 1990).

Growth factors such as EGF and PDGF can trigger a variety of cellular responses, including proliferation and cytoarchitectural changes, through binding to their specific receptors on the cell surface, thereby enabling transmembrane signaling (Ullrich and Schlessinger, 1990). These receptors have intracellular PTK domains and, upon ligand binding, are autophosphorylated on tyrosine residues. The phosphorylated tyrosines are subsequently recognized by signaling molecules, including phosphatidylinositol-3-kinase, phospholipase C-$\gamma$, ras GTPase-activating protein (Ras-GAP) and non-receptor PTKs of the Src family (Cantley *et al.*, 1991). Many of these molecules share the SH2 domain (Koch *et al.*, 1991; Pawson and Gish, 1992), which is capable of binding to phosphorylated tyrosine residues (PY) (Matsuda *et al.*, 1990; Waksman *et al.*, 1992), and, quite often, also contain the SH3 domain. Although the function of the SH3 domain has yet to be elucidated (Koch *et al.*, 1991; Pawson and Gish, 1992), considering the role of SH2 in binding to PY, SH3 may transduce signals to downstream target(s).

Recent studies have shown that among the molecules acting far downstream are small GTP-binding proteins, Ras for mitogenesis (Mulcahy *et al.*, 1985; Smith *et al.*, 1986; Yu *et al.*, 1988), Rac for membrane ruffle formation (Downward, 1992b; Ridley *et al.*, 1992) and Rho for actin stress fiber organization (Downward, 1992a; Ridley and Hall, 1992). One of the keys to the intracellular signaling mechanisms accordingly lies in identifying the molecules that link receptor PTKs and small GTP-binding proteins. Ash/Grb-2, which is composed of SH2 and SH3, is therefore a potential candidate for the linking protein.

In the present study an attempt has been made to confirm a role for Ash/Grb-2 in signaling by receptor PTKs by microinjecting an Ash-specific antibody into cells. We here report that an anti-Ash antibody abolishes the effects of EGF and PDGF not only on cell proliferation but also, unexpectedly, on membrane ruffle formation, which is regulated by the Rac function. The findings imply a role for Ash in both the signaling pathways from the receptor PTKs to Ras and Rac and, therefore, Ash may be key protein linking receptor PTKs and small GTP-binding proteins.

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## Results

### Association of Ash with EGF-R

Rabbit polyclonal antibodies were raised against an Ash peptide fused to glutathione S-transferase (GST) (Matuoka *et al.*, 1992). The antibody rA53f used in the present study was verified to detect Ash as a single band of ~28 kDa upon immunoblotting of whole cell lysates of both A-431 cells and NRK49F cells (Figure 1a). This indicates the specific recognition of Ash by the antibody (the weak signal at 49.5 kDa is most likely an IgG-related peptide detected in all immunoblotting and immunoprecipitation experiments using any antibody and preimmune IgG). Since Ash has been found to bind to receptor PTKs such as EGF receptor (EGF-R) and PDGF receptor (PDGF-R) (Lowenstein *et al.*, 1992; Rozakis-Adcock *et al.*, 1992), Ash association with EGF-R was confirmed by immunoprecipitation with an anti-EGF-R antibody. When A-431 cells were metabolically labeled with [$^{35}$S]methionine and treated with or without EGF, a 28 kDa protein was observed in anti-EGF-R antibody immunoprecipitates from EGF-stimulated cells. This signal was absent in immunoprecipitates from unstimulated cells (Figure 1b, top). The molecule was identified as Ash by parallel immunoblotting in which unlabeled cell lysates were treated with an anti-EGF-R antibody and immunoprecipitates were subjected to detection with an anti-Ash antibody (Figure 1b, bottom). Ash was also detected in anti-PY antibody immunoprecipitates from EGF-stimulated, but not unstimulated, cells. In addition, activated EGF-R after a denaturation (by SDS) and renaturation cycle, as well as non-denatured EGF-R, was capable of binding to an Ash fusion protein (Figure 1c). In contrast, the binding of other molecules to the Ash fusion protein (at approximately 55, 62, 84, 100, 135 and 160 kDa for EGF-stimulated A-431 cells and at 58 kDa for both stimulated and unstimulated cells) was either prevented or greatly decreased by such denaturation (with the exception of the 160 kDa protein). An anti-Ash antibody inhibited the binding of activated EGF-R to the Ash fusion protein. These observations imply a direct and strong association of Ash with activated EGF-R.

The association of Ash with EGF-R is therefore considered to be dramatically enhanced by EGF stimulation and to be of importance in signaling events downstream from EGF-R activation. If this is the case, interference with Ash function would affect EGF-triggered cellular events. This idea prompted us to examine the effect of an anti-Ash antibody on growth factor-induced mitogenesis.

### Inhibition of EGF- and PDGF-induced DNA synthesis by an anti-Ash antibody

In the presence of 0.1% platelet-poor plasma (PPP), serum-deprived NRK cells enter S phase ~9 h after exposure to

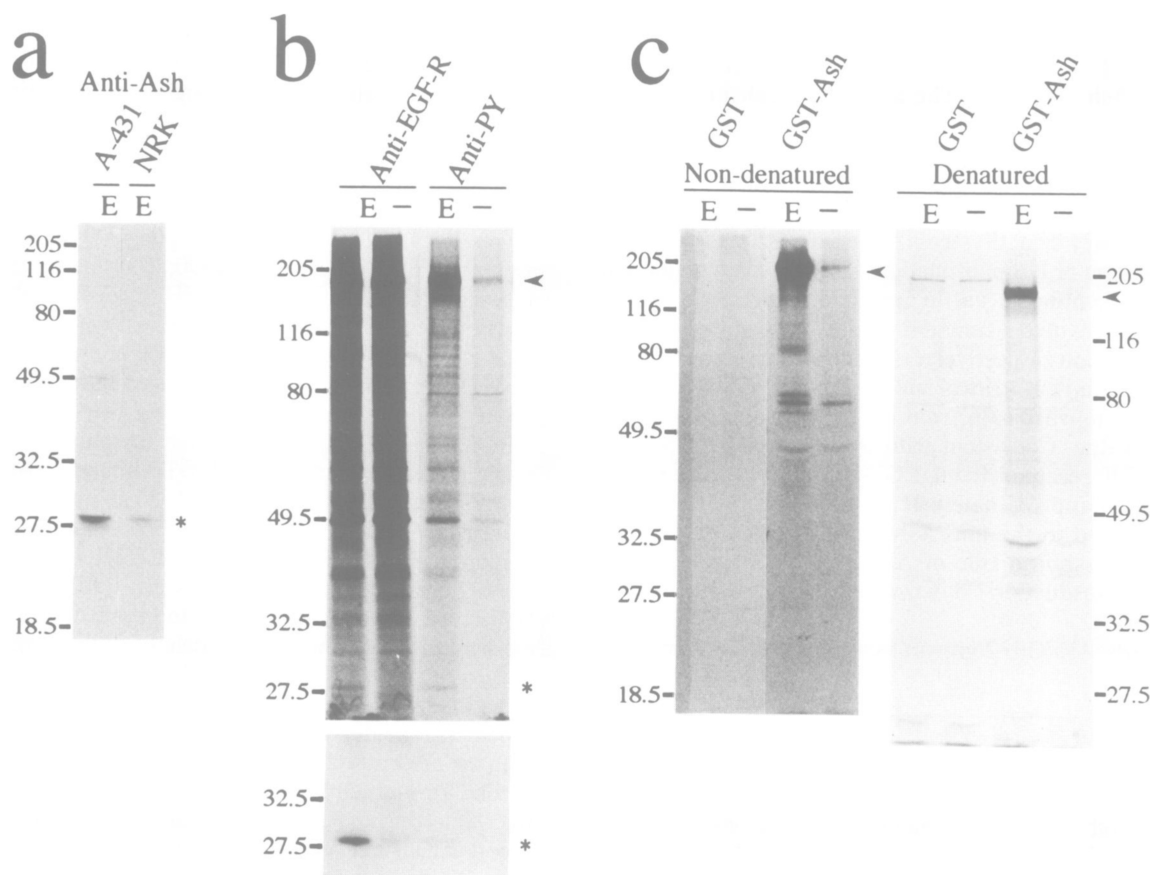


**Fig. 1.** Immunochemical detection of Ash in whole cell lysates and the *in vivo* and *in vitro* association of Ash with EGF-R. (**a**) Whole cell lysates from A-431 cells (500 μg protein) and NRK cells (200 μg protein) immunoblotted with anti-Ash antibody. (**b**) Immunoprecipitates from A-431 cell lysates with anti-EGF-R or anti-PY antibodies (top, prelabeled with [$^{35}$S]methionine and exposed for 4 days to visualize the 28 kDa band; bottom, immunoblotted with anti-Ash antibody, 6 mg of cellular protein subjected). (**c**) Whole cell lysates from [$^{35}$S]methionine-labeled A-431 cells (left: non-denatured; right: denatured and then renatured) were incubated with GST–Ash fusion protein or GST. Bound materials were autoradiographed (exposure for 2 days). –, no mitogen; E, EGF. Numbers show the position of molecular size markers (kDa) and asterisks indicate Ash.

EGF, PDGF and serum, but not to basic fibroblast growth factor (FGF) (Figure 2). Therefore, quiescent cells were microinjected with an anti-Ash antibody and exposed 1 h later to mitogens. The level of DNA synthesis was then determined 12–18 h after mitogenic stimulation. As shown in Figure 3, anti-Ash antibody injection brought about a decrease in S phase entry induced by EGF and PDGF to the level of unstimulated cells. The antibody was effective in bringing about this decrease at 1, 2 and 4 mg/ml, but had only a marginal effect at 0.5 mg/ml (not shown). In contrast, serum-induced DNA synthesis was only slightly affected. Since the antibody used recognizes Ash specifically (Figure 1a) and since the injection of preimmune IgG (Figure 3) or buffer alone (not shown) had no effect, the abolition of EGF- and PDGF-induced mitogenesis can be ascribed to an action of the antibody, most likely an impairment in Ash function caused by binding to endogenous Ash molecules. Ash may therefore be indispensable for signal transduction downstream from the EGF-R and PDGF-R.

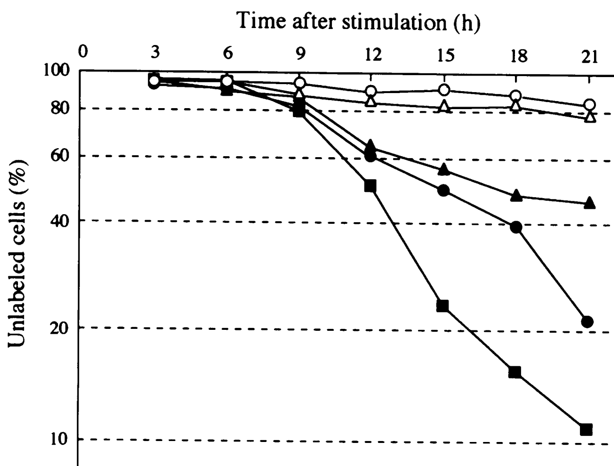


**Fig. 2.** S phase entry of NRK cells exposed to mitogens. Cells were serum-deprived in PPP-supplemented medium for 18–24 h, stimulated with mitogens at time zero and incubated for the indicated periods. The percentage of cells not synthesizing DNA during the incubation (unlabeled cells) was scored (averages of 3–5 samples). ○, no mitogen; △, FGF; ▲, EGF; ●, PDGF; and ■, serum.

***Effect of an anti-Ash antibody on reorganization of cytoskeleton***

In the course of experiments involving anti-Ash antibody injection, we noticed a substantial difference in morphology between injected and non-injected cells. We therefore examined the effect of the antibody on cytoarchitecture. When NRK cells are serum-deprived in the presence of 0.1% PPP, they remain rich in actin stress fibers with smooth pericellular actin construction and few membrane ruffles or pseudopodia. Exposure of PPP-incubated cells to EGF and PDGF elicits pericellular roughening, pericellular actin accumulation, membrane ruffling and a substantial decrease in stress fibers within 10 min (Figure 4a). After 18 h exposure to the stimulants, in addition to the above effects, pseudopodium formation and a change in cell shape are apparent (Figure 4b). An anti-Ash antibody, when injected 1 h prior to stimulation with EGF or PDGF, prevents this reorganization (Figure 4a). The antibody also causes a reversion in actin fiber assembly in EGF- and PDGF-stimulated cells to the level of that in unstimulated (i.e. PPP-incubated) cells (Figure 4b). Parallel results were obtained by injecting the anti-Ash antibody into PPP-deprived cells (Figure 4c), except that the cells were deficient in stress fibers in the absence of PPP. As a control, preimmune rabbit IgG or buffer alone was injected in an identical manner, the results being the same as for uninjected samples (not shown).

Similar experiments were performed with various stimulation and injection protocols. The results can be summarized as follows: (i) the absence of any stimulant results in a substantial decrease in the number of stress fibers and the amount of roughening of pericellular actin construction (as seen in Figure 4c), upon which the injection of anti-Ash antibody has no effect; (ii) serum, PPP and lysophosphatidic acid (LPA) induce stress fibers, and anti-Ash antibody fails to suppress this induction regardless of whether it is injected before or after stimulation; (iii) basic FGF has no effect on the cytoarchitecture of NRK cells treated or untreated with other stimulants; (iv) EGF and PDGF elicit pericellular actin accumulation and membrane ruffle/pseudopodium formation, any of which are prevented by injection of an anti-Ash antibody either before or after stimulation; and (v) exposure of PPP- or LPA-pretreated cells (which hence are rich in stress fibers) to EGF and

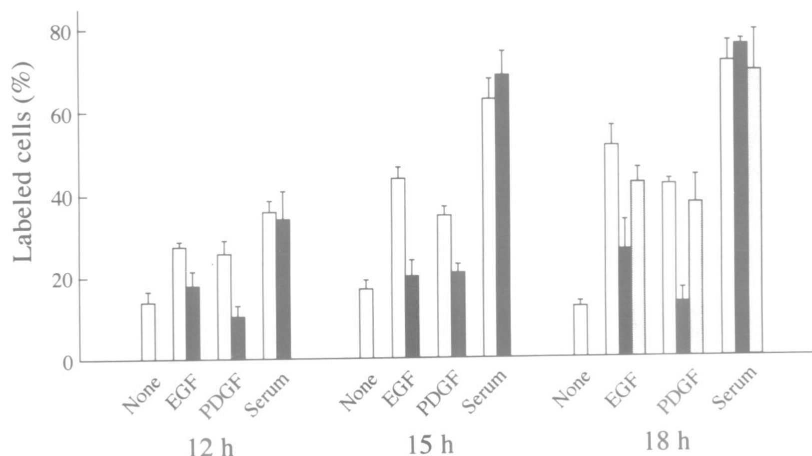


**Fig. 3.** S phase entry of NRK cells injected with anti-Ash antibody. Percentage of cells synthesizing DNA was determined at the indicated times after growth factor stimulation. Short bars indicate standard errors of the mean ($n$ = 3–5). Open bars, uninjected; filled bars, injected with anti-Ash antibody; dotted bars, injected with preimmune rabbit IgG.

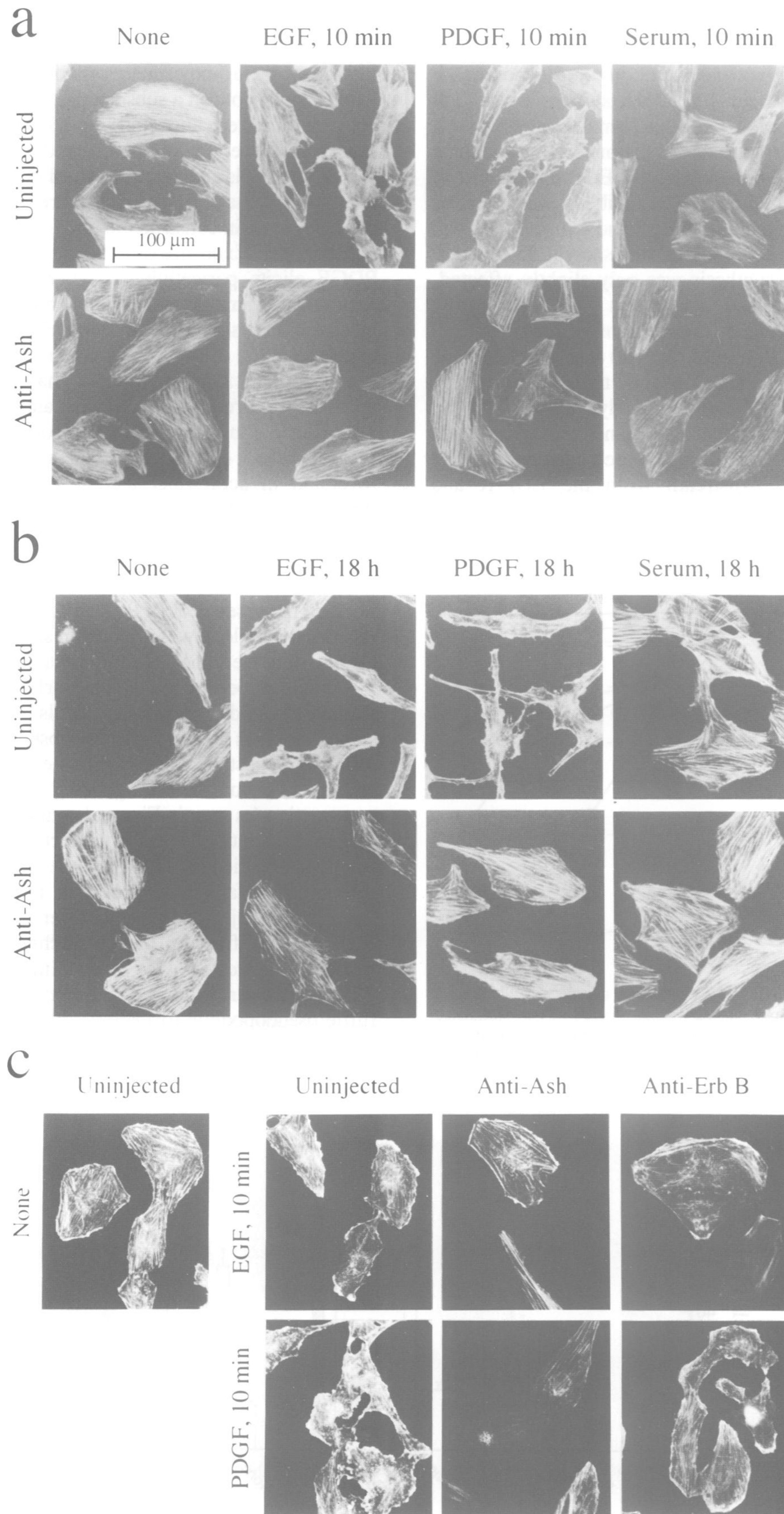


**Fig. 4.** Changes in the actin fiber assembly of NRK cells due to stimulation and antibody injection. (**a**) Cells were serum-deprived in a PPP-supplemented medium, injected with anti-Ash antibody, incubated for 1 h and exposed to stimulants for 10 min. (**b**) Cells were exposed to stimulants for 17 h in the presence of PPP, injected with anti-Ash antibody and incubated for an additional 1 h. (**c**) Cells were serum-deprived in the absence of PPP, injected with anti-Ash and anti-Erb B antibodies, incubated for 1 h and exposed to stimulants for 10 min.

PDGF decreases the number of stress fibers (dramatically in PPP-treated cells and moderately in LPA-treated cells), and this stress fiber reduction is absent from cells injected with the anti-Ash antibody before exposure to stimulants.

As recently demonstrated, the reorganization of actin fiber assembly is under the control of the Rho and Rac proteins, the former being indispensable for the formation of stress fibers (Ridley and Hall, 1992) and the latter for the formation of ruffles (Ridley *et al.*, 1992). The results of the present experiments indicate that Ash may function in Rac-related signaling pathways downstream from EGF-R and PDGF-R. Consistent with this idea, identical results were obtained by injecting a polyclonal antibody directed against the v-*erb* B gene product (having a broad reactivity with vertebrate c-Erb B, and hence EGF-R; Carpenter and Cohen, 1979) (Figure 4c). An anti-Erb B antibody suppressed actin assembly reorganization upon subsequent exposure to EGF. The antibody had little, if any, effect on PDGF-induced cytoskeletal changes. Next, we microinjected an anti-Ras antibody to clarify whether or not membrane ruffling is induced through Ras activation. Anti-Ras antibody did not inhibit PDGF-induced membrane ruffling (not shown), suggesting that membrane ruffling is caused by signaling pathways independently of Ras activation in NRK cells.

The present findings show that, at least in NRK cells, Ash plays a role not only in Ras-related signaling pathways, but also in those involved in membrane ruffling, suggesting that, apart from the signaling molecules for Ras, those for Rac also associate with Ash.

### *Associated proteins with Ash*

In a search for possible candidates for the downstream signal transducers, [$^{35}$S]methionine-labeled cells were subjected to anti-Ash antibody immunoprecipitation (Figure 5). When lysates of A-431 cells were treated with an anti-Ash antibody, a number of molecules, in addition to the strong signal at 28 kDa for Ash, were detected in the immunoprecipitates (Figure 5a). Unexpectedly, only a very faint, if any, signal was detected at 180 kDa, the position corresponding to EGF-R. So far, repetitions of the experiments and comparison with mock samples treated with preimmune IgG have confirmed that only a few bands represent anti-Ash antibody-specific co-precipitates. In both EGF-stimulated and unstimulated cells, strong signals were obtained at approximately 160, 135, 62 and 55 kDa, with a weaker signal at ~21 kDa. An additional 29 kDa band was detected only in EGF-treated cells. The 160 and 135 kDa proteins might be identical to those shown to bind to the Ash fusion protein *in vitro* (Figure 1c).

When lysates of NRK cells (Figure 4b) and rat 3Y1 cells (not shown) were immunoprecipitated with the anti-Ash antibody, 160 and 135 kDa proteins were co-precipitated regardless of EGF stimulation. For all cell types examined, the major proteins associated with Ash had molecular masses of 160 and 135 kDa. The amounts of 160 and 135 kDa proteins associated with Ash appeared to be unaffected by mitogen stimulation. In contrast, the signal for the 29 kDa molecule in NRK cells was strengthened by EGF stimulation. With 3Y1 cells, in which PDGF, but not EGF, exerts mitogenesis, an increase in the 29 kDa signal was observed only upon PDGF stimulation (not shown). In neither of these cell types did serum stimulation cause a change of the signal strength for the 29 kDa molecule.

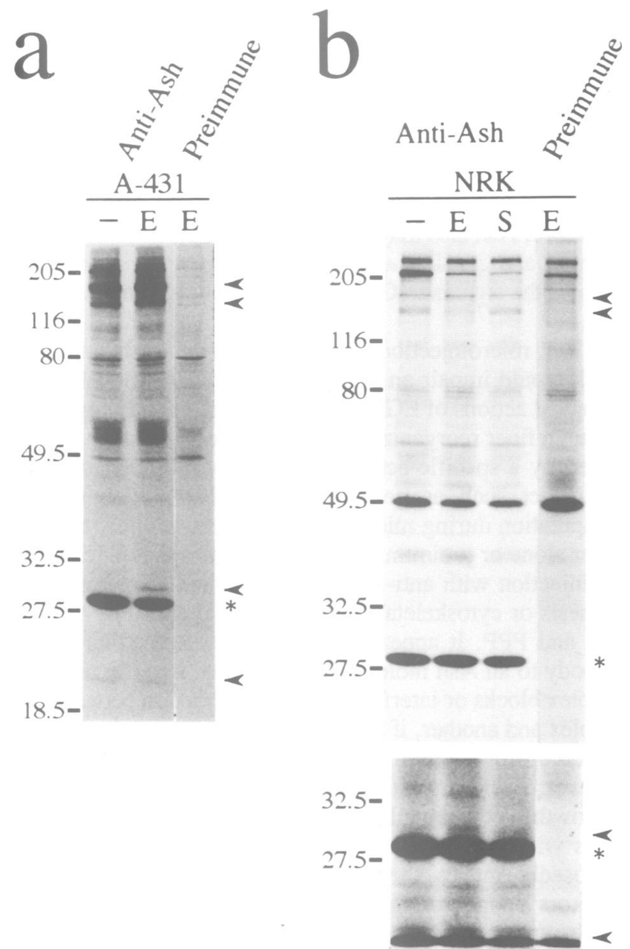


**Fig. 5.** Molecules recovered in anti-Ash antibody immunoprecipitates. (**a**) Anti-Ash antibody immunoprecipitates from [$^{35}$S]methionine-labeled A-431 cell lysates (exposure for 2 days). (**b**) Anti-Ash antibody immunoprecipitates from [$^{35}$S]methionine-labeled NRK cell lysates (top, exposure for 2 days; bottom, exposure for 8 days to visualize the 29 and 21 kDa bands). –, no mitogen; E, EGF; S, serum. Asterisks indicate Ash and arrowheads Ash-binding molecules (see text).

## Discussion

In the present study the function of Ash/Grb-2 was investigated using an Ash-specific antibody for microinjection and immunoprecipitation. Since Ash is the mammalian homolog of a nematode signaling protein, Sem-5, which functions between a receptor PTK and Ras protein (Clerk *et al.*, 1992), it was predicted that Ash would play a role similar to Sem-5 in mammalian cells.

The presence of Ash in anti-EGF-R antibody immunoprecipitates appears to be one of the most notable differences between EGF-treated and untreated cells. This implies that Ash association with EGF-R is one of the major events following the ligand activation of EGF-R and is accordingly important to the subsequent signaling process. In addition, activated EGF-R is still capable of binding to an Ash fusion protein after denaturation/renaturation, which supposedly disrupts intermolecular interactions and dissociates bound proteins from one another. This observation suggests that Ash couples directly with activated EGF-R or that it is a signaling protein next to EGF-R. Taken together with other reports (Lowenstein *et al.*, 1992; Rozakis-Adcock *et al.*,

1992), Ash is likely to be a key molecule acting downstream from such receptor PTKs as EGF-R and PDGF-R.

More recently, the *Drosophila ash/grb-2* (*drk*) gene has been cloned (Olivier *et al.*, 1993; Simon *et al.*, 1993) and it has been demonstrated that the Drk protein binds the Sevenless tyrosine kinase with its SH2 domain and the *sos* guanine nucleotide-releasing protein through its SH3 domain. Therefore, Ash also may be present between tyrosine kinase receptors and guanine nucleotide-releasing proteins, and may transmit the signals to GTP-binding proteins in mammalian cells.

In fact, microinjection of cells with an anti-Ash antibody, which would impair an Ash function *in vivo*, abolishes the biological actions of EGF and PDGF, including mitogenesis and actin fiber reorganization. These effects must have been caused by a specific action of the antibody rather than by an artifact such as general cellular dysfunction due to manipulation during microinjection, because injection with buffer alone or preimmune IgG caused no apparent changes and injection with anti-Ash antibody failed to affect DNA synthesis or cytoskeletal reorganization induced by serum, LPA and PPP. It appears possible that the binding of the antibody to an Ash molecule incorporated into the receptor complex blocks or interferes with an interaction between the complex and another, if any, key signaling molecule, leading to a failure in signal transduction. The anti-Ash antibody, however, exerts its effects even when microinjected prior to growth factor stimulation. This means that the antibody reacts with Ash molecules uncoupled from receptors and supposedly makes them unable to associate with their receptors upon subsequent ligand activation.

The anti-Ash antibody also has an effect on the cytoarchitecture reorganization induced by EGF and PDGF, but again, not on that induced by serum: EGF and PDGF induce pericellular accumulation of actin fibers and membrane ruffling accompanied by a decrease in actin stress fibers in 10 min and, in the case of long-term exposure to growth factors for 18 h, pseudopodium formation. This reorganization of the cytoarchitecture is abolished by microinjecting anti-Ash antibody regardless of whether the cells are injected before or after growth factor stimulation. In contrast, serum, LPA and PPP induce or increase actin stress fibers, an effect that is not sensitive to anti-Ash antibody.

A function of the Rac protein has been established to be indispensable for the above reorganization induced by EGF, PDGF and other stimulants employing a PTK-type receptor (Downward, 1992b; Ridley *et al.*, 1992), although the signaling pathways from the receptors to Rac have yet to be clarified. On the other hand, stress fiber formation has been found to depend on a Rho function and to be induced by serum, LPA and bombesin (Downward, 1992b; Ridley and Hall, 1992), with LPA and bombesin both employing a trimeric G protein system (van Corven *et al.*, 1989; Battey *et al.*, 1991). The anti-Ash antibody discrimination of Rho-dependent events from those dependent on Rac may be explained in a parallel manner to its effects on growth factor-promoted mitogenesis as discussed above, provided that the active substances in serum and PPP are compounds like LPA and phosphatidic acid. The indication here is again that Ash may play a role in signaling pathways originating from receptor PTKs, but, in this instance, down to the Rac protein.

Ash has been reported to bind to a 55 kDa protein (Lowenstein *et al.*, 1992) and to 46, 52 and 66 kDa subtypes of Shc (Rozakis-Adcock *et al.*, 1992) in a tyrosine phosphorylation-dependent manner. In our experiments, it is not likely that molecules with corresponding molecular masses associate with Ash and its fusion peptide in response to EGF stimulation. But, since we hardly detected activated EGF-R in the immunoprecipitates with anti-Ash antibody, only a small part of Ash seems to bind EGF-R and Shc in a tyrosine phosphorylation-dependent manner, resulting in a failure to detect Shc in the immunoprecipitates.

The present study has demonstrated the possibility that Ash not only plays a role in Ras-related signaling pathways but also functions for Rac regulation downstream of receptor PTKs, at least in NRK cells, though we did not obtain direct evidence that Rac is present downstream of Ash. Further studies are required to establish the molecular interactions around the receptor PTKs and Ash. Still, the findings reported here suggest *in vivo* interactions of Ash with multiple molecules that may regulate both Ras and Rac functions. The identification of molecules that interact with Ash and the elucidation of Ash function can be expected to unravel the complex signaling strings linking small GTP-binding proteins.

## Materials and methods

### *Antibodies and cells*

A GST fusion protein (Smith and Johnson, 1988) of Ash (amino acids 15–217) was prepared (Matuoka *et al.*, 1988) and used to immunize rabbits. The polyclonal antibodies raised were purified by adsorption with a lysate of GST-expressing bacteria and successive binding to the GST–Ash fusion protein and protein A. One lot of antibodies, referred to as rA35f, was used for the present study. Other antibodies used were anti-EGF-R [Ab-1, mouse monoclonal antibody (mAb) to human EGF-R; Oncogene Sciences], anti-PY (PY-20, mouse mAb to synthetic phosphotyrosine; ICN ImmunoBiologicals), anti-v-Erb B (rabbit polyclonal antibody to bacterially expressed v-*erb* B gene product; Medac) and anti-v-H-Ras (Ab-1, rat mAb to v-H-Ras; Oncogene Sciences). The following cultured cells were all obtained from the Japanese Cancer Research Resources Bank: A-431 human epidermoid carcinoma cells (Giard *et al.*, 1973), NRK49F normal rat kidney-derived fibroblasts (De Larco and Todaro, 1978) and 3Y1 rat fibroblasts (Kimura *et al.*, 1975). Unless otherwise stated, cells were cultured in Dulbecco's modification of Eagle's Minimum Essential Medium (DME; Gibco) supplemented with 10% fetal bovine serum (HyClone). For experiments, the cells were deprived of serum in medium containing 0.1% horse PPP (Sigma) for 18–24 h and then exposed to the following stimulants: 0.1% PPP, 5% serum, 100 ng/ml of murine EGF (Gibco), 10 ng/ml of recombinant human BB type PDGF (Gibco), 20 ng/ml of recombinant human basic FGF (Gibco) and 200 ng/ml oleoyl L-α-LPA (Sigma).

### *Immunoprecipitation and fusion protein binding*

Cells were deprived of serum in PPP-supplemented medium and exposed to growth factors for 10 min as described above. For metabolic labeling with [$^{35}$S]methionine, the same medium except for the absence of methionine (Met-free DME; Gibco) was used. Four hours prior to stimulation, [$^{35}$S]methionine (ICN Biomedicals) was added (10 MBq per 60 mm dish). Whole cell lysates were prepared by scraping cells directly in SDS–PAGE sample buffer. For immunoprecipitation experiments, cells were scraped and sonicated in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.5 mM $Na_3VO_4$, 1% Triton X-100, 100 μM phenylmethylsulfonyl fluoride, 100 μM diisopropyl fluorophosphate and 10 μg/ml aprotinin) and the lysates were cleared by centrifugation at 100 000 *g* for 1 h. The lysates were then incubated first with protein A–agarose (Pierce) bearing preimmune rabbit IgG at room temperature for 2–3 h and then with protein A–agarose bearing antibodies to Ash, EGF-R or PY at 4°C overnight. The beads were washed five times in lysis buffer and boiled in SDS–PAGE sample buffer. SDS–PAGE was performed in 8–12% gels, which were then subjected to immunoblotting with the Western-Light System (Tropix) or immersed in Enlightening (New England Nuclear) and dried for autoradiography. To examine *in vitro*

binding, [$^{35}$S]methionine-labeled cell lysates, either not denatured, or denatured by boiling in 2% SDS solution for 10 min and then renatured by diluting to bring the SDS concentration to 0.1%, were incubated with glutathione−Sepharose (Pharmacia) bearing ~50 μg of GST−Ash or GST at 4°C overnight and processed for autoradiography as described above.

### *Antibody microinjection and DNA synthesis determination*

NRK cells were deprived of serum in medium supplemented with 0.1% PPP and 2 mg/ml anti-Ash antibody or preimmune rabbit IgG in 140 mM potassium phosphate, pH 7.25, was microinjected into the cytoplasm of the cells by the method described previously (Matuoka *et al.*, 1988). In control experiments, 0.5, 1 and 4 mg/ml antibody were also used. One hour after injection, the cells were exposed to either EGF, PDGF or serum and cultured in the presence of 50 μM 5-bromo-2′-deoxyuridine for the indicated periods. The proportion of cells synthesizing DNA was determined using a BrdU Detection Kit (Boehringer Mannheim).

### *Examination of actin fiber assembly*

Cells were deprived of serum in the presence or absence of PPP, injected with anti-Ash or anti-Erb B antibodies as described above, incubated for 1 h and exposed to stimulants for 10 min. Alternatively, serum-deprived cells in parallel cultures were exposed to stimulants for 17 h (in the presence or absence of PPP), injected with the anti-Ash antibody and incubated in stimulant-containing medium for an additional 1 h. Cell staining procedures consisted of fixation in 3.7% formalin in phosphate buffered saline at room temperature for 20 min, extraction in 0.5% Triton X-100 at room temperature for 10 min, blocking in 50 μg/ml bovine γ-globulin in Tris-buffered saline containing 0.1% Tween 20 at 4°C overnight and staining with 3.3 nM rhodamine-labeled phalloidin (Molecular Probes) at 37°C for 1 h.

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