Characterization of the intracellular signalling pathways that underlie growth-factor-stimulated glucose transport in *Xenopus* oocytes: evidence for *ras*- and *rho*-dependent pathways of phosphatidylinositol 3-kinase activation

Fiona J. THOMSON*‡, Thomas J. JESS*, Colin MOYES*, Robin PLEVIN† and Gwyn W. GOULD*

*Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K. and †Department of Physiology and Pharmacology, University of Strathclyde, George Street, Glasgow G1 1XW, Scotland, U.K.

The stimulation of glucose transport is one of the early cellular responses to growth factors and is essential for cell proliferation, yet the molecular processes that underlie this response are poorly defined. The aim of this study was to characterize the role of the low-molecular-mass G-proteins, Ras and Rho, and their downstream targets, Raf protein kinase and phosphatidylinositol 3-kinase, in the regulation of glucose transport in *Xenopus* oocytes by two distinct growth-factor receptors: the insulin-like growth factor I (IGF-I) tyrosine kinase receptor and the heterotrimeric G-protein-coupled lysophosphatidic acid (LPA) receptor. Micro-injection of a neutralizing anti-Ras antibody partially blocked IGF-I-stimulated deoxyglucose uptake but was without effect on LPA-stimulated deoxyglucose uptake. In contrast, micro-injection of the C3 coenzyme of botulinum toxin, which selectively ADP-ribosylates and inactivates Rho, inhibited LPA-stimulated, but not IGF-I-stimulated, deoxyglucose uptake. Similarly, LPA- but not IGF-I-stimulated deoxyglucose uptake was attenuated in oocytes expressing a dominant negative *rho*

construct. Cells expressing a dominant negative mutant of Raf protein kinase exhibited markedly reduced sensitivity to both LPA and IGF-I, consistent with a role for endogenous Raf in glucose uptake by both growth factors. Furthermore, expression of a constitutively activated form of *raf-1* resulted in a growth-factor-independent increase in deoxyglucose uptake. Measurements of phosphatidylinositol 3-kinase activity in microinjected cells support the hypothesis that the IGF-I receptor stimulates glucose transport by a Ras-dependent activation of phosphatidylinositol 3-kinase, whereas the G-protein-coupled LPA receptor controls this response by a pathway that involves Rho-dependent activation of a distinct phosphatidylinositol 3-kinase. Thus we provide evidence for clear differences in the signalling pathways that control glucose transport by G-protein-coupled and tyrosine kinase growth-factor receptors. Furthermore this is the first demonstration that active Rho is involved in the signalling pathways that regulate glucose uptake in response to some growth factors.

INTRODUCTION

Exposure of resting cells to growth factors causes activation of numerous signal-transduction pathways which culminates several hours later in cell growth and division. Cells undergoing mitogenesis have an increased energy requirement and thus stimulation of glucose transport is a common action of all mitogens. The time course of mitogen-stimulated glucose transport is biphasic; an acute response occurs within minutes which is followed by a chronic effect after approx. 4–12 h [1–6]. The acute stimulation of glucose uptake by mitogens is thought to result from the translocation of intracellular glucose transporters to the cell surface [7,8], whereas the chronic effect is associated with increased glucose transporter gene transcription and protein synthesis [1–5,8,9]. However, the growth-factor-activated second-messenger pathways that mediate these changes in cellular glucose uptake are poorly understood.

In an attempt to elucidate the intracellular signalling pathways that underline the acute stimulation of glucose transport in cells, we have utilized oocytes isolated from *Xenopus laevis*. Oocytes provide a useful system for the study of receptor-stimulated signalling events that lead to glucose transport, since they exhibit a 2–3-fold increase in deoxyglucose (dGlc) uptake in response to distinct growth-factor receptors: the tyrosine kinase insulin-like growth factor I (IGF-I) receptor and the heterotrimeric G-protein-linked lysophosphatidic acid (LPA) receptor [6,10,11]. In

previous studies using this system, we have identified a crucial role for phosphatidylinositol 3-kinase (PI 3-kinase) activation in growth-factor-stimulated dGlc transport [11,12]. However, our experiments indicate that distinct PI 3-kinase forms participate in the control of sugar transport by LPA and IGF-I. This situation may reflect differences in the pathways that couple these different receptors to their distinct PI 3-kinase isoforms. To test this hypothesis, we have attempted to identify the growth-factor-stimulated pathways that lead to activation of these distinct PI 3-kinases.

Biochemical analysis has shown that the low-molecular-mass G-proteins, p21^{ras} and p21^{rho} can regulate PI 3-kinase activity, perhaps by directly binding to the regulatory subunit of PI 3-kinase [13,14]. Furthermore both LPA and IGF-I receptors couple to signal-transduction pathways that lead to activation of Ras, Rho and Raf protein kinase (see [15,16] for reviews), and previous investigations suggest that Ras and Raf may regulate glucose transport into cells. In 3T3-L1 adipocytes, for example, expression of constitutively active mutants of *ras* and *raf* leads to an increase in glucose transport and an alteration in the cellular distribution of GLUT1 [17–19]. The involvement of the Rho family of low-molecular-mass G-proteins in the pathways that regulate glucose uptake has not yet been determined. However, Rho is believed to play a major role in growth-factor-induced cytoskeletal reorganization [20] and therefore is a suitable candidate effector of glucose-transporter translocation.

To investigate the involvement of Ras, Rho and Raf in growth-factor-stimulated dGlc transport, we have examined the effects of neutralizing antibodies and of expression of dominant negative and constitutively active mutants. Microinjection of neutralizing anti-Ras antibodies into *Xenopus* oocytes partially inhibited IGF-I-stimulated dGlc uptake, but had no effect on sugar uptake in response to LPA. Furthermore IGF-I-stimulated, but not LPA-stimulated, PI 3-kinase activation was markedly reduced in oocytes injected with anti-Ras antibody. In contrast, LPA-stimulated [^3H]dGlc uptake and PI 3-kinase activation was attenuated in oocytes expressing a dominant negative mutant of Rho or in oocytes that had been microinjected with botulinum toxin C3 coenzyme, which inactivates Rho. Expression of a constitutively active Raf kinase construct resulted in a significant increase in oocyte dGlc uptake, whereas expression of a dominant negative *raf* construct inhibited the stimulation of dGlc uptake by both IGF-I and LPA. Therefore Raf may be common to the pathways that stimulate glucose transport by both growth factors. However, PI 3-kinase activation by both of these growth factors was not affected by expression of either constitutively active or dominant negative *raf* in oocytes. These results suggest that a Ras-dependent activation of PI 3-kinase may underlie the stimulation of glucose transport in response to IGF-I, whereas a Rho-dependent activation of PI 3-kinase may mediate this response to LPA. Thus differences exist in the signal-transduction pathways that lead to the acute increase in glucose uptake by tyrosine kinase and G-protein-coupled growth-factor receptors.

MATERIALS AND METHODS

Materials

Female wild-caught *Xenopus laevis* were purchased from the South African Xenopus Facility (Noordhoek, R.S.A.). IGF-I was from Boehringer-Mannheim (Lewes, East Sussex, U.K.), and LPA and PtdIns were from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Cytochalasin B was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). [$2,6\text{-}^3\text{H}$]dGlc (8.1 Ci/mmol) was purchased from DuPont-NEN (Stevenage, Herts., U.K.) and [$\gamma\text{-}^{32}\text{P}$]ATP from Amersham International (Little Chalfont, Bucks., U.K.). Rat monoclonal anti-Ras (Y13 259) was purchased from Affiniti Ltd. (Bristol, U.K.). C3 coenzyme was purchased from LC laboratories, Nottingham, U.K. The dominant negative and truncated *c-raf* cDNA constructs were generously provided by Professor Chris Marshall (Chester Beatty Laboratories, London, U.K.). The dominant negative *rho* cDNA construct was generously provided by Dr Gabor Tigyi (University of Tennessee, Memphis, TN, U.S.A.). All other reagents were as described [6,21].

Isolation of oocytes from *Xenopus laevis*

The isolation and microinjection of oocytes was performed exactly as described previously [21,22]. Stage-V and -VI oocytes were isolated using watchmaker's forceps and incubated in Barth's buffer [5 mM Hepes/NaOH, pH 7.4, 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO_3 , 0.82 mM MgSO_4 , 0.41 mM CaCl_2 , 0.33 mM $\text{Ca}(\text{NO}_3)_2$]. For studies using neutralizing Ras antibody, C3 toxin and cRNA Raf-1 and Rho mutants, oocytes were microinjected as described [21,22]. Before experimentation, damaged oocytes were removed.

Sugar-transport assays in oocytes

The transport of dGlc was assayed as described previously [21,22]. Briefly, groups of 10 oocytes were incubated in 0.2 ml of Barth's buffer in 13.5 ml centrifuge tubes. Transport measure-

ments were initiated by the addition of an aliquot of [$2,6\text{-}^3\text{H}$]dGlc (1 μCi , 50 μM per assay). After incubation at room temperature for the indicated times, the reaction was stopped by rapid aspiration of the medium and washing of the oocytes with 5 ml of ice-cold PBS. Oocytes were washed in this fashion twice more and dispensed to scintillation vials (one oocyte per vial) containing 1.0 ml of 1% SDS. The vials were incubated with agitation before scintillant was added and radioactivity was measured. The amount of sugar non-specifically associated with the oocytes was accounted for by performing duplicate uptake measurements in the presence of 10 μM cytochalasin B, a potent inhibitor of facilitative glucose transporters. We have previously shown that under these conditions, transport of dGlc and not its subsequent phosphorylation is rate-limiting [22].

cDNA subcloning and synthesis of cRNA *in vitro*

Constitutively activated *raf*(Δraf) was constructed by deletion of amino acids 1–324 whereas a dominant negative *raf*(ΔNraf) defective in the ATP-binding site was created by Lys-375 $\rightarrow$ Trp-375 [23]. The structure of dominant negative *rho* (ΔNrho) was based on wild-type Rho A, but had residue 19 exchanged for an asparagine, locking the protein into an inactive GDP-bound form [24]. cDNA constructs were subcloned into the vector pGEM-4Z (Promega, Southampton, Hants., U.K.), and cRNA was transcribed *in vitro*, essentially as described previously, using linearized plasmid DNA as a template [21].

Measurement of oocyte PI 3-kinase activity

Oocytes were incubated in Barth's buffer at room temperature, with or without 1-stearoyl LPA (100 μM) or IGF-I (250 nM). At the indicated times, the medium was aspirated, and the oocytes were washed six times in 4 ml of 20 mM Tris/HCl (pH 7.2)/140 mM NaCl. Oocytes were resuspended in 20 mM Tris/HCl, pH 7.2, containing 140 mM NaCl, 1 mM sodium orthovanadate, 10% (v/v) glycerol, 0.5 mM dithiothreitol, 0.1 μM pepstatin A, 2 mM di-isopropyl fluorophosphate, 200 μM aprotinin and 0.1 mM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (30 oocytes/600 μl) and homogenized by 20 strokes of a Dounce homogeniser. The homogenate was centrifuged at 100 000 g, 4 $^\circ\text{C}$, 60 min and the supernatant was snap-frozen in liquid N_2 and stored at $-80\text{ }^\circ\text{C}$ before assay.

PI 3-kinase activity in oocyte homogenates was assayed by the phosphorylation of PtdIns with [^{32}P]ATP as previously described [11,25–27]. Reactions (final volume 50 μl) were terminated by the addition of 80 μl of 1 M HCl, and the lipids were extracted with 180 μl of chloroform/methanol (1:1, v/v). Extracted lipids (50 μl) were spotted on a 5 cm $\times$ 20 cm silica-gel 60 plate (Whatman International Ltd., Maidstone, Kent, U.K.) and phosphatidylinositol phosphates were separated on the ability of phosphatidylinositol 4-phosphate, but not phosphatidylinositol 3-phosphate, to form a borate complex with the *cis*-diol present in the inositol ring as previously described [28]. The position of the phosphatidylinositol 3-phosphate spot was determined using a ^{32}P -labelled phosphatidylinositol 3-phosphate standard followed by autoradiography. The corresponding section of the plate was excised and the ^{32}P incorporated was measured by scintillation counting.

Statistical analysis

Statistical analysis of results was performed using StatView Software (Abacus) running on a Macintosh computer.

RESULTS

Role of Ras in IGF-I- and LPA-stimulated glucose transport in oocytes

In previous studies, expression of a constitutively active mutant of *ras* in 3T3-L1 adipocytes has been shown to cause a growth-factor-independent increase in glucose transport [17–19]. Furthermore LPA and IGF-I can activate Ras in other cells (see [15,16] for reviews; [29]). However, to investigate whether active Ras is required for glucose transport in response to growth factors, we examined the effect of microinjection of neutralizing anti-Ras antibodies [30] on both IGF-I- and LPA-stimulated dGlc transport in oocytes (Figure 1). Injection of anti-Ras antibodies consistently resulted in a reduction in dGlc uptake stimulated by IGF-I, but this inhibition was significant only at higher concentrations of agonist. Interestingly, IGF-I-stimulated dGlc transport was not completely inhibited using this approach. It is not clear whether this represents an inability of the antiserum to effectively inhibit all of the Ras protein present in an oocyte, or whether this represents an element of Ras-independent signalling from the IGF-I receptor in this system. However, injection of increasing amounts of antibody failed to result in any further reduction of the IGF-I response (Figure 1, top), suggesting that a Ras-independent pathway may also be required for stimulation of glucose uptake. In contrast, within the same preparation of oocytes, microinjection of anti-Ras antibodies failed to inhibit LPA-stimulated dGlc transport (Figure 1, bottom). These data argue that only certain growth-factor receptors stimulate glucose transport by coupling to a Ras-dependent mechanism whereas others couple to a Ras-independent process.

Role of Rho in IGF-I- and LPA-stimulated glucose transport in oocytes

There is evidence to suggest that the LPA receptor may mediate certain cellular responses by coupling to the Rho family of low-molecular-mass G-proteins [20,31]. Certainly, evidence suggests that Rho is a key regulator of the actin cytoskeleton (see [32] for review) and thus may be involved in the trafficking of GLUT1 from an intracellular location to the plasma membrane. To investigate whether active Rho is necessary for the action of either LPA or IGF-I on glucose uptake in oocytes, we expressed a dominant-negative *rho* construct (Δ Nrho), which acts by competitively inhibiting the interaction of endogenous Rho with its effectors [24] (Figure 2). IGF-I-stimulated dGlc uptake in oocytes expressing Δ Nrho was not significantly different from the same response in control oocytes (Figure 2, top). In contrast, LPA-stimulated dGlc uptake was significantly reduced by expression of Δ Nrho, suggesting the involvement of a Rho-dependent pathway of glucose uptake by this growth factor (Figure 2, bottom). Microinjection of antisense cRNA had no significant effect on responses to either growth factor (Figure 2). Microinjection of oocytes with the C3 coenzyme from botulinum toxin, which selectively ADP-ribosylates and inactivates Rho [33,34], inhibited LPA-stimulated, but not IGF-I-stimulated, dGlc uptake in oocytes (Table 1). These data further substantiate the hypothesis that active Rho is required for LPA- but not IGF-I-stimulated dGlc transport.

Role of Raf kinase in the regulation of glucose transport by LPA and IGF-I

Biochemical and genetic studies have identified Raf protein kinase as a direct target of Ras [35–37] and is perhaps the best characterized downstream effector of Ras-dependent signalling.

Figure 1 Effect of microinjection of anti-Ras antibodies on IGF-I- and LPA-stimulated dGlc transport in oocytes

Oocytes were microinjected with anti-Ras antibody such that the intracellular concentrations corresponded to dilutions of 1:50 (▲) or 1:5 (●), or with random mouse IgG at a serum dilution of 1:5 (control, ■). After a 5 h incubation at 18 °C, oocytes were exposed to IGF-I (top) or LPA (bottom) for 60 min at the concentrations shown. After this time, dGlc uptake was assayed as described in the Materials and methods section. Results are means $\pm$ S.E.M. for three independent experiments and have been corrected for non-specific association of isotope with the oocytes (typically less than 10% of total uptake) by performing parallel assays in the presence of 10 μ M cytochalasin B, a potent inhibitor of facilitative glucose transporters.

* Statistically significant inhibition compared with control ($P < 0.05$).

Figure 2 Effect of expression of dominant negative *rho* on IGF-I- and LPA-stimulated dGlc transport in oocytes.

Oocytes were microinjected with 50 nl of cRNA (~1 mg/ml), encoding either sense or antisense dominant negative *rho*, or water (control oocytes). After an overnight incubation at 18 °C, oocytes were exposed to IGF-I (top) or LPA (bottom) for 60 min at the concentrations shown. After this time, dGlc uptake was assayed as described in the Materials and methods section. Results are means ± S.E.M. for three independent experiments and have been corrected for non-specific association of isotope with the oocytes by performing parallel assays in the presence of 10 μM cytochalasin B, a potent inhibitor of facilitative glucose transporters. * Statistically significant inhibition compared with control ($P < 0.05$).

Table 1 Effect of microinjection of C3 coenzyme on LPA- and IGF-I-stimulated dGlc uptake into oocytes

Oocytes were microinjected with 20 nl of either water or a 4 mg/ml stock of C3 coenzyme and incubated in Barth's buffer for 30 min. Groups of 10 oocytes were then incubated for an additional 30 min with IGF-I (250 nM) or LPA (100 μM) before assay of dGlc transport as described in the Materials and methods section. The data are from a representative experiment using 10 oocytes (mean ± S.E.M.), repeated for each agonist twice more with similar results. * Statistically significant difference from corresponding water-injected group ($P < 0.05$).

	[³ H]dGlc uptake (pmol/h per oocyte)	
	Water-injected	C3 coenzyme
Basal	7.2 ± 0.6	9.6 ± 1.3
LPA (100 μM)	15.1 ± 1.1	6.2 ± 0.4*
IGF-I (250 nM)	18.5 ± 0.9	20.3 ± 1.3

Table 2 Effect of expression of a constitutively activate *raf* construct on dGlc uptake into oocytes

Oocytes were microinjected with 50 nl of water, sense or antisense Δ raf cRNAs (each at ~1 mg/ml) and incubated for 24–36 h in Barth's buffer. Groups of 10 oocytes were then assayed for basal dGlc transport as described in the Materials and methods section. The data are from a representative experiment and are means ± S.E.M. for 10 oocytes, repeated twice with similar results. * Statistically significant difference between corresponding control groups ($P < 0.05$).

	[³ H]dGlc uptake (pmol/h per oocyte)	
	Water-injected	Δ raf sense
Water-injected	7.6 ± 0.5	19.1 ± 1.6*
Δ raf antisense	7.5 ± 0.6	

Expression of a constitutively active *raf* mutant in fibroblasts and adipocytes has been shown previously to lead to an increase in basal glucose uptake [17–19], suggesting that Raf may indeed have a role in the signalling pathways that control GLUT1-mediated glucose transport. For this reason, we set out to examine the role of Raf in growth-factor action in *Xenopus* oocytes.

Expression of a constitutively active *raf* (Δ Raf) construct in oocytes resulted in a growth-factor-independent increase in basal dGlc uptake, whereas expression of an antisense construct had no effect (Table 2). These results are consistent with a role for Raf kinase in the control of glucose transport in oocytes by growth factors. Expression of a dominant negative *raf* construct (Δ Nraf) in *Xenopus* oocytes resulted in a significant reduction in both LPA- and IGF-I-stimulated dGlc uptake over a wide range of growth-factor concentrations. In addition, there was a small rightward shift in the concentration–response curves for both IGF-I- and LPA-stimulated dGlc uptake (Figure 3). In contrast, microinjection of antisense cRNA had no effect on either basal or growth-factor-stimulated dGlc transport (results not shown). These data suggest that active Raf is required for the increase in glucose uptake into oocytes in response to LPA and IGF-I receptor activation. However, we cannot dismiss the possibility that Δ Nraf expression might inhibit growth-factor-stimulated glucose uptake, not by interfering with the activation of wild-type Raf, but by sequestering another essential component of the pathway, e.g. Ras, Rho.

Figure 3 Effect of expression of dominant negative *raf* on IGF-I- and LPA-stimulated dGlc transport in oocytes

Oocytes were microinjected with 50 nl of water or cRNA (~50 ng) encoding dominant negative *raf*. After an overnight incubation at 18 °C, oocytes were exposed to IGF-I (top) or LPA (bottom) for 60 min. After this time, dGlc uptake was assayed as described in the Materials and methods section. Results are means $\pm$ S.E.M. for three independent experiments and have been corrected for non-specific association of isotope with the oocytes (typically less than 10% of total uptake) by performing parallel assays in the presence of 10 μ M cytochalasin B, a potent inhibitor of facilitative glucose transporters. * Statistically significant inhibition compared with control ($P < 0.05$).

Table 3 Effect of microinjection of anti-Ras antibodies on IGF-I- and LPA-stimulated PI 3-kinase activity in oocytes

Oocytes were microinjected with 50 nl of anti-Ras antibody or random mouse IgG (control) (effective intracellular antibody concentration corresponding to a 1:5 dilution of serum). After a 5 h incubation at 18 °C, oocytes were exposed to IGF-I (250 nM) or LPA (100 μ M) after which homogenates were prepared as described in the Materials and methods section. PI 3-kinase activity was measured by the phosphorylation of PtdIns as described in the Materials and methods section. Results are means $\pm$ S.E.M. for four independent experiments. * Statistically significant inhibition compared with control ($P < 0.05$).

	PI 3-kinase activity (fmol/ μ g of protein)	
	Control	Anti-Ras
Basal	17.9 $\pm$ 0.9	18.3 $\pm$ 1.6
LPA (100 μ M)	35.0 $\pm$ 1.8	30.0 $\pm$ 3.0
IGF-I (250 nM)	30.9 $\pm$ 1.2	21.5 $\pm$ 2.6*

Table 4 Effect of expression of dominant negative *rho* on IGF-I- and LPA-stimulated PI 3-kinase activity in oocytes

Oocytes were microinjected with 50 nl of approx. 1 mg/ml Δ NRho cRNA or water as a control. After an overnight incubation at 18 °C, oocytes were exposed to IGF-I (250 nM) or LPA (100 μ M) after which homogenates were prepared as described in the Materials and methods section. PI 3-kinase activity was measured by the phosphorylation of PtdIns as described in the Materials and methods section. Data are from a representative experiment and are means $\pm$ S.E.M.; the experiment was repeated twice with similar results. * Statistically significant inhibition compared with control ($P < 0.05$).

	PI 3-kinase activity (fmol/ μ g of protein)	
	Control	Δ NRho
Basal	18.9 $\pm$ 1.0	25.2 $\pm$ 1.2
LPA (100 μ M)	45.6 $\pm$ 1.9	21.4 $\pm$ 1.7*
IGF-I (250 nM)	33.2 $\pm$ 1.6	42.1 $\pm$ 2.6

Role of Ras and Rho in the mechanism of PI 3-kinase activation by LPA and IGF-I

We have previously shown that activation of PI 3-kinase is crucial for the stimulation of glucose transport in oocytes by IGF-I and LPA [11,12]. However, our previous experiments also indicate that distinct PI 3-kinase forms regulate glucose uptake by these two growth factors [11]. It is also probable that there are differences in the mechanisms by which the G-protein-coupled LPA receptor and the tyrosine kinase IGF-I receptor couple to these distinct PI 3-kinases. To test the possibility that this difference may lie in the ability of these two growth factors to couple to different low-molecular-mass G-proteins, we measured LPA- and IGF-I-stimulated PI 3-kinase activity in oocytes that had been microinjected with either anti-Ras antibodies (Table 3) or cRNA encoding dominant negative Rho (Table 4). IGF-I-stimulated PI 3-kinase activity was significantly inhibited in anti-Ras-injected oocytes, although not to basal levels. In contrast, LPA-stimulated PI 3-kinase was unaffected by antibody microinjection (Table 3).

The effects of expression of dominant negative Rho on both LPA- and IGF-I-stimulated PI 3-kinase activities are presented in Table 4. Expression of dominant negative Rho clearly inhibited LPA-stimulated PI 3-kinase, but was without effect on IGF-I-stimulated PI 3-kinase. These data provide evidence that active

Table 5 Effect of expression of dominant negative Raf on IGF-I- and LPA-stimulated PI 3-kinase activity in oocytes

Oocytes were microinjected with 50 nl of approx. 1 mg/ml Δ Nraf cRNA. After an overnight incubation at 18 °C, oocytes were exposed to IGF-I (250 nM) or LPA (100 μ M) after which homogenates were prepared as described in the Materials and methods section. PI 3-kinase activity was measured by the phosphorylation of PtdIns as described in the Materials and methods section. Results are means $\pm$ S.E.M. for three independent experiments. * Statistically significant inhibition compared with control ($P < 0.05$).

	PI 3-kinase activity (fmol/ μ g of protein)	
	Control	Δ Nraf
Basal	19.2 $\pm$ 0.7	21.3 $\pm$ 2.0
LPA (100 μ M)	34.0 $\pm$ 1.3	31.7 $\pm$ 1.4
IGF-I (250 nM)	52.7 $\pm$ 3.2	53.5 $\pm$ 2.4

Ras lies upstream on PI 3-kinase after IGF-I receptor activation, whereas PI 3-kinase activation by the LPA receptor requires active Rho.

Role of Raf in the mechanism of PI 3-kinase activation by LPA and IGF-I

Given that our data indicate that Rho and Ras are required for the control of glucose uptake by the LPA and IGF-I receptors respectively and that Raf and PI 3-kinase may both lie downstream of Ras and/or Rho, we next set out to investigate the relationship between Raf and PI 3-kinase activation after growth-factor stimulation of oocytes. Consequently, we examined the effect of expression of dominant negative *raf* mutant (Δ Nraf) on LPA- and IGF-I-stimulated PI 3-kinase activity in oocytes (Table 5). Both LPA- and IGF-I-stimulated PI 3-kinase activation were unaffected by expression of Δ Nraf in oocytes (Table 5). Furthermore expression of a constitutively active *raf* construct failed to have any significant effect on oocyte PI 3-kinase activity (21 ± 2.6 fmol/ μ g in control oocytes compared with 19.0 ± 2.1 fmol/ μ g in Δ Nraf-cRNA-injected oocytes). It is possible therefore that PI 3-kinase and Raf may lie on distinct signal-transduction pathways which both serve to regulate glucose transport. Alternatively, PI 3-kinase may lie upstream of Raf in the second-messenger cascades that regulate sugar uptake.

DISCUSSION

Studies of the acute effects of growth factors on glucose transport have yet to reach a consensus regarding the important intracellular signalling pathways that are required for this response. The LPA and IGF-I growth-factor receptors are structurally dissimilar and signal through distinct intracellular pathways, the LPA receptor being G-protein-coupled [38–41] whereas the IGF-I receptor belongs to the tyrosine kinase receptor family [42]. Nevertheless, short-term exposure of cells to these growth factors results in the same effect; a 2–3-fold increase in glucose-transport rate. Since these two growth factors have similar effects on glucose transport, it would seem likely that the intracellular signals generated by each will converge at a common point. In our previous investigations, we have provided evidence that suggests that activation of PI 3-kinase is common to the control of sugar transport by LPA and IGF-I. However, these two receptor types appear to control glucose uptake via distinct PI 3-kinase forms [11,12], and it is probable that this reflects the ability of these distinct receptors to couple to different pathways

upstream of PI 3-kinase activation. The data provided here support this concept. We have shown that microinjection of oocytes with a monoclonal antibody that interferes with the function of Ras *in vivo* had no effect on LPA-stimulated dGlc uptake or PI 3-kinase activation, but inhibited IGF-I-stimulated dGlc transport and PI 3-kinase activation. In contrast, ablation of Rho function, either with C3 toxin or by expression of a dominant negative *rho* mutant, attenuated LPA- but not IGF-I-stimulated glucose uptake and PI 3-kinase activation. Thus LPA-stimulated glucose transport is dependent on active Rho upstream of PI 3-kinase, whereas the IGF response requires active Ras for PI 3-kinase activation. Previous studies have indicated that activation of low-molecular-mass G-proteins and PI 3-kinase are intimately linked in receptor-regulated signalling cascades [43,44]; however, this investigation illustrates the importance of this pathway in the regulation of a physiologically relevant response. Furthermore this is one of the first demonstrations that Rho is involved in the signalling pathways that regulate glucose transport, at least by certain growth factors.

In addition to PI 3-kinase, GTP-bound p21^{ras} is capable of interacting with and activating a number of signalling proteins, including Ral-GDS, protein kinase C ζ , Raf-1, A-Raf and B-Raf [35], (see [45] for a review), and it could be that any of these may also serve to regulate glucose transport. Indeed, previous studies have shown that expression of oncogenic Raf in fibroblasts results in elevated GLUT1 mRNA and protein levels and increased glucose uptake [19]. Consistent with these data, we have shown that expression of a constitutively active *raf* construct in oocytes causes an increase in glucose transport (Table 2), indicating a role for Raf in the regulation of glucose uptake. However, these studies do not address the role of Raf in growth-factor-activated signalling pathways. Moreover, in these studies, Raf expression is achieved by incubating cRNA-injected oocytes for 24 h before measuring glucose transport. It is possible that the effect of active Raf is to increase cellular glucose uptake, not by mimicking the acute growth-factor response by altering the subcellular localization of GLUT1, but by causing a concurrent increase in glucose-transport protein levels. However, the very low levels of glucose-transporter protein in oocytes preclude further analysis to confirm this hypothesis.

We subsequently investigated the involvement of Raf in growth-factor-stimulated dGlc uptake by studying the effect of expression of a dominant negative *raf* construct (Δ Nraf) in oocytes. In these studies, both LPA- and IGF-I-stimulated dGlc uptake were attenuated in oocytes expressing Δ Nraf. This decrease in transport rate may either result from a reduction in transporter number and/or by interference of the Δ Nraf mutant with the pathways that cause transporter translocation. However, the data in Figure 3 show that Δ Nraf expression has no significant effect on basal levels of dGlc uptake, suggesting that expression of this mutant has little, if any, effect on the level of transporter expression. Thus a Raf-dependent pathway may be required for the stimulation of glucose transport in response to IGF-I and LPA. Further attempts to define the role of Raf in growth-factor action by measuring Raf activity were precluded by technical problems, since oocytes only contain very small amounts of Raf protein and commercially available Raf antibodies do not reliably immunoprecipitate oocyte Raf.

The observation that Raf, but not Ras, is involved in the regulation of glucose transport by LPA is somewhat interesting, since this would suggest that activation of Raf by the LPA receptor occurs by a Ras-independent process. Other G-protein receptors have been demonstrated to stimulate Raf-1 in a Ras-independent manner [46,47], and Raf-1 can directly interact with G-protein $\beta\gamma$ -subunits [48], although a subsequent change in

Raf-1 kinase activity has yet to be demonstrated. A similar mechanism may account for Ras-independent activation of Raf-1 by the LPA receptor. However, the possibility exists that Raf may not be directly involved in growth factor action and that the mutant Raf protein is inhibiting glucose uptake by interfering with endogenous Ras, Rho or another unidentified effector, such that their ability to signal to their downstream targets is prevented. Consistent with this hypothesis, the ability of the p120^{as} GTPase-activating protein to bind to and stimulate Ras-GTP hydrolysis is inhibited by Raf-1 [36,37]. However, since both LPA- and IGF-1-stimulated PI 3-kinase activity were both unaffected by expression of the dominant negative Raf, then it would seem that mutant Raf is not interfering with the signalling pathways that lead to PI 3-kinase, at least. Thus the possibility that a Raf-1-dependent pathway is involved in growth-factor regulation of glucose transport should not be completely overlooked.

Regulated exocytosis, such as is observed for the movement of glucose transporters to the plasma membrane after growth factor stimulation, requires specialized proteins that bestow specificity to the targeting event, thus controlling membrane fusion. The evidence presented in this study suggests that diverse signalling pathways involving activation of PI 3-kinase/Rho and PI 3-kinase/Ras can contribute to LPA- and IGF-I-stimulated glucose uptake respectively, perhaps by co-operating in the translocation of intracellular vesicles containing sugar transporters to the plasma membrane. Clearly, a number of important questions still remain to be addressed. For example, further studies are required to determine the molecular basis of PI 3-kinase action on glucose-transporter function. Furthermore other growth-factor-receptor-activated signalling cascades in addition to the Rho- and Ras-dependent pathways are required for the regulation of glucose transport. Thus a complex array of different signals is likely to co-ordinate the control of glucose uptake. As our knowledge of these signalling pathways unfolds, then our understanding of the molecular mechanisms that underlie growth-factor action, and thus proliferative diseased states, will increase.

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