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The heterotrimeric G_q protein-coupled angiotensin II receptor activates $p21^{ras}$ via the tyrosine kinase-Shc-Grb2-Sos pathway in cardiac myocytes

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$p21^{ras}$ plays an important role in cell proliferation, transformation and differentiation. Recently, the requirement of $p21^{ras}$ has been suggested for cellular responses induced by stimulation of heterotrimeric G protein-coupled receptors. However, it remains to be determined how agonists for G protein-coupled receptors activate $p21^{ras}$ in metazoans. We show here that stimulation of the G_q protein-coupled angiotensin II (Ang II) receptor causes activation of $p21^{ras}$ in cardiac myocytes. The $p21^{ras}$ activation by Ang II is mediated by an increase in the guanine nucleotide exchange activity, but not by an inhibition of the GTPase-activating protein. Ang II causes rapid tyrosine phosphorylation of Shc and its association with Grb2 and mSos-1, a guanine nucleotide exchange factor of $p21^{ras}$. This leads to translocation of mSos-1 to the membrane fraction. Shc associates with the SH3 domain of Fyn whose tyrosine kinase activity is activated by Ang II with a similar time course as that of tyrosine phosphorylation of Shc. Ang II-induced increase in the guanine nucleotide exchange activity was inhibited by a peptide ligand specific to the SH3 domain of the Src family tyrosine kinases. These results suggest that an agonist for a pertussis toxin-insensitive G protein-coupled receptor may initiate the cross-talk with non-receptor-type tyrosine kinases, thereby activating $p21^{ras}$ using a similar mechanism as receptor tyrosine kinase-induced $p21^{ras}$ activation.

Keywords: angiotensin II/Fyn/G_q protein/guanine nucleotide exchange activity/$p21^{ras}$

Introduction

The *ras* proto-oncogenes encode closely related proteins of relative molecular mass around 21 000 ($p21^{ras}$). $p21^{ras}$ plays an important role in various signaling pathways for cellular growth, differentiation and transformation (Satoh *et al.*, 1992; Boguski and McCormick, 1993). The activity of $p21^{ras}$ is regulated through binding of guanine nucleotides to $p21^{ras}$. Active $p21^{ras}$ is bound to GTP, whereas inactive $p21^{ras}$ is bound to GDP. The ratio of GTP/GDP bound to $p21^{ras}$ is regulated by both guanine nucleotide exchange factors, which catalyze GDP release and GTP binding, and the GTPase-activating protein (GAP), which stimulates the intrinsic $p21^{ras}$ GTPase activity (Satoh *et al.*, 1992; Boguski and McCormick, 1993). It has been shown that the active $p21^{ras}$ associates with other intracellular signaling molecules, such as Raf-1 and phosphatidylinositol-3 kinase, thereby regulating the downstream protein kinase cascades, including the mitogen-activated protein (MAP) kinase kinase (MEK)–MAP kinase–90 kDa S6 kinase (RSK) cascade (Blenis, 1993; Avruch *et al.*, 1994; Rodriguez-Viciana *et al.*, 1994).

Activation of $p21^{ras}$ has been shown to occur after treatment of cells with a variety of stimuli, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin (Satoh *et al.*, 1992). These growth factors activate receptor tyrosine kinases which regulate $p21^{ras}$ activity by stimulating a transient interaction between the receptor and the guanine nucleotide exchange factor mSos-1 via the adapter protein Grb2 having Src homology (SH)2/SH3 domains (Buday and Downward, 1993a; Egan *et al.*, 1993). Although it is generally appreciated that activation of $p21^{ras}$ by growth factors is mediated by a tyrosine kinase-dependent mechanism, there are other mechanisms as well. For example, antigen-induced activation of $p21^{ras}$ in T cells is mediated primarily by inhibition of GAP activity through a protein kinase C (PKC)-dependent mechanism (Downward *et al.*, 1990). Recently, agonists for the pertussis toxin-sensitive G protein (G_i type)-coupled receptors, such as muscarine, thrombin and lysophosphatidic acid receptors, have also been shown to activate $p21^{ras}$ (Alblas *et al.*, 1993; Cook *et al.*, 1993; van Corven *et al.*, 1993; Winitz *et al.*, 1993; Hordijk *et al.*, 1994). Some agonists (thyrotropin-releasing hormone and epinephrine) for pertussis toxin-insensitive G protein (such as G_q)-coupled receptors can also activate $p21^{ras}$ (Koch *et al.*, 1994; Ohmichi *et al.*, 1994). Furthermore, the requirement of $p21^{ras}$ has been shown in mitogenic response initiated by G protein-coupled receptors, such as thrombin and thyrotropin (LaMorte *et al.*, 1993; al-Alawi *et al.*, 1995). However, heterotrimeric G protein-coupled receptors neither possess intrinsic protein tyrosine kinase activity nor have known physical association with other tyrosine kinases (Neer, 1995). Importantly, these receptors stimulate phospholipases Cβ, D and A_2, and produce various lipid-derived second messengers that have been shown to inhibit GAP activity *in vitro* (Tsai *et al.*, 1989). Therefore, it remains to be determined whether $p21^{ras}$ activation by G protein agonists is mediated by stimulation of the guanine nucleotide exchange activity or by inhibition of GAP activity. Moreover, the signal transduction mechanism of $p21^{ras}$ activation by G protein-coupled receptor agonists remains to be elucidated.

$p21^{ras}$ may also play a critical role in post-mitotic cells such as cardiac myocytes. Thorburn *et al.* (1993) have reported that microinjection of a dominant negative $p21^{ras}$ mutant into cultured cardiac myocytes prevents stimulation of the atrial natriuretic factor promoter by the α_1 adrenergic receptor agonist phenylephrine, suggesting that $p21^{ras}$

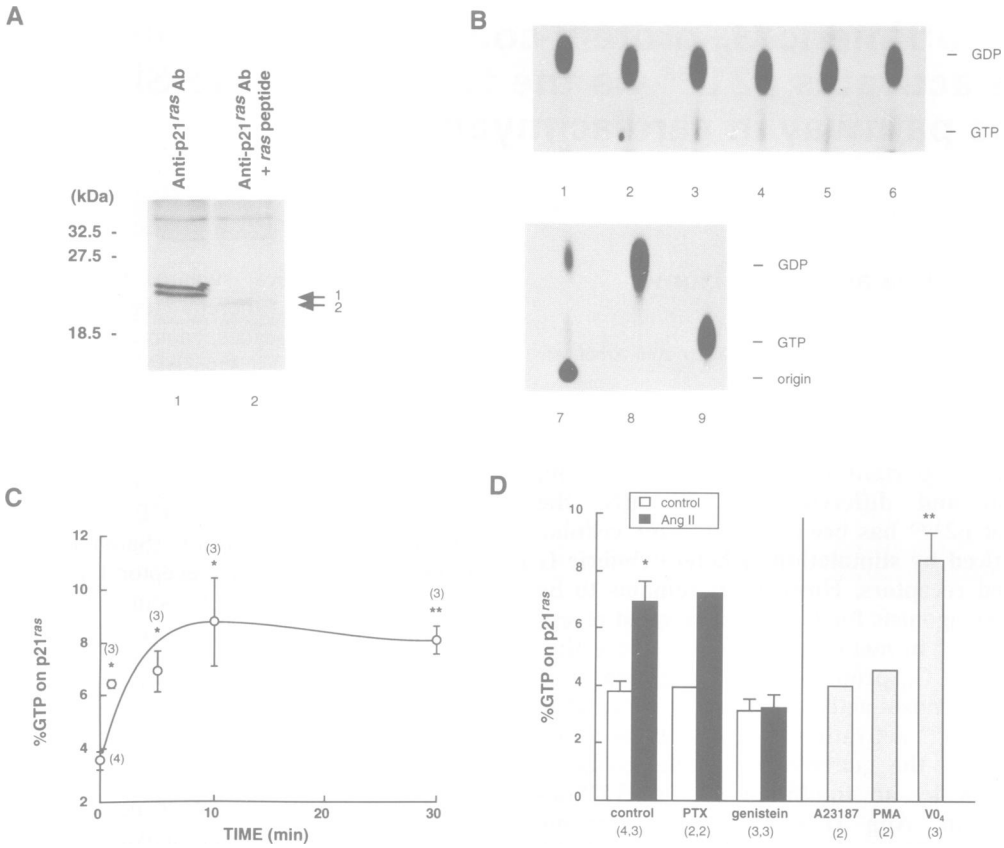

Fig. 1. Activation of p21^{ras} by Ang II in cardiac myocytes. (**A**) Existence of two forms of p21^{ras} in cardiac myocytes. Myocytes were labeled with [^{35}S]methionine. Immunoprecipitation was performed using an anti-p21^{ras} antibody (Y13-259). In lane 2, the anti-p21^{ras} antibody was pre-incubated with antigen peptide for 1 h before immunoprecipitation. Immunoprecipitates were subjected to SDS–PAGE on a 15% gel. The results shown are representative of four independent experiments. Ab, antibody. Molecular size standards are indicated at the left in kDa. (**B**) Cardiac myocytes were labeled with [^{32}P]orthophosphate and stimulated with Ang II (100 nM) for 0 (lane 1), 5 (lanes 2 and 5), 10 (lanes 3 and 7) or 30 (lane 4) min, or with fetal bovine serum (20%) for 10 min (lane 6). In lane 5, myocytes were pre-treated with pertussis toxin (500 ng/ml for 24 h) and then stimulated with Ang II. p21^{ras} was immunoprecipitated and bound guanine nucleotides were separated by TLC. Standard [^{3}H]GDP (2 μCi) and [α-^{32}P]GTP (4 nCi) were also subjected to TLC, and the spots obtained are shown in lanes 8 and 9, respectively. The β emission of [^{3}H]GDP was enhanced. The results are representative of four (control) and two (pertussis toxin pre-treatment) independent experiments. (**C**) Time course of p21^{ras} activation in response to Ang II in cardiac myocytes. The results are the mean ± SE obtained from 3–4 samples. (**D**) Effects of various drugs on p21^{ras} activation. Some myocytes were pre-treated with genistein (30 μM) for 30 min or pertussis toxin (500 ng/ml) for 24 h and then stimulated with Ang II (100 nM) for 5 min. Some myocytes were stimulated with PMA (1 μM), A23187 (30 μM) or pervanadate (VO_4, 50 μM) for 5 min. The stock solution of pervanadate was prepared by mixing 5 vols of 10 mM Na orthovanadate with 1 vol. of 500 mM hydrogen peroxide. Results shown are the mean + SE from 2–4 separate experiments. *$P < 0.05$, **$P < 0.01$ versus control without any drugs.

may play an important role in the phenylephrine-induced hypertrophic response of cardiac myocytes. Interestingly, mechanical loading, the most important physiological stimulus for cardiac hypertrophy, of neonatal rat cardiac myocytes in culture rapidly activates p21^{ras} (Sadoshima and Izumo, 1993a). We have recently shown that mechanical loading (stretch) causes an autocrine secretion of angiotensin II (Ang II) and Ang II plays a critical role in mediating stretch-induced cardiac hypertrophy (Sadoshima and Izumo, 1993b; Sadoshima *et al.*, 1993). However, it is not known whether Ang II, which couples to G_q or G_i, or any other agonists for G protein-coupled receptors that cause cardiac hypertrophy activate p21^{ras}. Therefore, in this study we asked whether Ang II activates p21^{ras} and, if so, what is the signal transduction mechanism leading to p21^{ras} activation. Our results suggest that stimulation of the Ang II receptor causes activation of the Src family kinases and tyrosine phosphorylation of the adapter protein Shc, with the subsequent recruitment of Grb2–Sos1 complex to the membrane fraction to activate p21^{ras}. These results provide the first evidence that signal transduction leading to p21^{ras} activation by a heterotrimeric G_q protein-coupled receptor shares a common pathway with that utilized by tyrosine kinase receptors and cytokine receptors.

Results

Angiotensin II activates p21^{ras} in cardiac myocytes

We first examined the expression of p21^{ras} in cultured cardiac myocytes. Immunoprecipitation after ^{35}S labeling of cardiac myocytes indicated that the anti-p21^{ras} antibody (Y13-259), which reacts with rodent K- and H-p21^{ras} (Furth *et al.*, 1982), specifically detected two bands around 21 kDa (Figure 1A, lane 1). These bands were not observed when the antibody was pre-absorbed with p21^{ras}-antigen peptide (Figure 1A, lane 2). It has been shown that K-p21^{ras} migrates more slowly than H-p21^{ras} on SDS–PAGE (Downward *et al.*, 1990). When we used another anti-p21^{ras} antibody (Y13-238), which specifically reacts

with rodent H-p21^{ras} (Furth *et al.*, 1982), a single band co-migrating with the lower band was observed (data not shown). This suggests that the upper band (band 1 in Figure 1A) most likely corresponds to K-p21^{ras} and the lower band (band 2 in Figure 1A) to H-p21^{ras}. In the remaining study, we used Y13-259 for immunoprecipitations and our results most likely reflect the properties of K- and H-p21^{ras}.

We next examined guanine nucleotide binding to p21^{ras}. Myocytes were stimulated with Ang II (100 nM) and cell lysates were subjected to immunoprecipitation with the anti-p21^{ras} antibody. Guanine nucleotides bound to the p21^{ras} immunoprecipitates were separated by TLC. Unstimulated cardiac myocytes kept in serum-free media for 48 h had low baseline levels of GTP-bound p21^{ras} (3.6 ± 0.4%, n = 4), which were comparable with those of NIH 3T3 fibroblasts (4.0 ± 0.6%, n = 3). Guanine nucleotides eluted from immunoprecipitates with the p21^{ras} antibody pre-absorbed with p21^{ras} antigen peptide had only background levels of ^{32}P activity (data not shown), indicating that the GTP and GDP radioactivities detected in TLC were from p21^{ras}. Ang II treatment significantly increased the ratio of GTP to GDP binding by 2.5-fold at 10 min (8.9 ± 1.6%, n = 3) and the increase in GTP binding persisted for >30 min (Figure 1B and C). The activation of p21^{ras} by Ang II was almost comparable with that by fetal bovine serum (9.0 ± 0.5% at 10 min, n = 3; Figure 1B, lane 6).

Angiotensin II-induced activation of p21^{ras} is insensitive to pertussis toxin but requires tyrosine kinase activity

To examine whether the p21^{ras} activation by Ang II is mediated by a pertussis toxin-sensitive G protein, such as $G_{i\alpha}$, the effect of Ang II on guanine nucleotide binding of p21^{ras} was examined after treating myocytes with pertussis toxin (500 ng/ml) over 24 h, a treatment which has been shown to fully ADP ribosylate the α subunit of G_i in this preparation (Allen *et al.*, 1988). The Ang II-induced increase in the ratio of GTP binding was not affected by the pertussis toxin treatment (Figure 1B, lane 5, and Figure 1D), indicating that the Ang II-induced p21^{ras} activation is not mediated by pertussis toxin-sensitive G proteins, such as G_i or G_o.

Ang II stimulation of cardiac myocytes causes a rapid activation of PKC (Sadoshima and Izumo, 1993c). Direct stimulation of PKC by phorbol 12-myristate 13-acetate (PMA) caused little increase in GTP-bound p21^{ras} (Figure 1D), suggesting that activation of PKC alone cannot account for Ang II-induced p21^{ras} activation. Ang II stimulation of cardiac myocytes also causes an elevation of intracellular Ca^{2+} (Sadoshima *et al.*, 1995). However, the Ca^{2+} ionophore A23187 failed to activate p21^{ras} (Figure 1D), indicating that elevation of intracellular Ca^{2+} is not sufficient to cause p21^{ras} activation. Ang II stimulation also causes a rapid activation of cellular tyrosine kinase activity in cardiac myocytes (Sadoshima *et al.*, 1995). Stimulation of tyrosine kinases by the tyrosine phosphatase inhibitor vanadate was sufficient to cause p21^{ras} activation and a tyrosine kinase inhibitor genistein inhibited Ang II-stimulated p21^{ras} activation (Figure 1D). These results suggest that tyrosine kinase activity is essential for Ang II-induced p21^{ras} activation.

Angiotensin II stimulates guanine nucleotide exchange activity but does not change GAP activity

Activation of p21^{ras} can be mediated either by activation of guanine nucleotide exchange activity or by inhibition of the p21^{ras} GTPase activity (Boguski and McCormick, 1993). To examine the mechanism of Ang II-induced p21^{ras} activation, we measured the guanine nucleotide exchange activity and the GAP activity in cardiac myocytes stimulated with Ang II. Guanine nucleotide exchange activity was measured using the permeabilized cell assay (Downward *et al.*, 1990). As shown in Figure 2A, although a significant baseline level of the guanine nucleotide exchange activity was observed in unstimulated myocytes, Ang II treatment (circles) further increased the exchange activity over unstimulated control (squares). Almost all of the radioactivity of the guanine nucleotides detected was from p21^{ras} because its level was near background when the p21^{ras} antibody in the immunoprecipitation procedure was pre-absorbed with p21^{ras} antigen peptide (triangles).

GAP activity was measured by incubating cell lysates with recombinant p21^{ras} complexed with [α-^{32}P]GTP and by quantifying the proportion of GTP-bound p21^{ras} (Downward *et al.*, 1990). As shown in Figure 2B, the lysates from control (squares) and Ang II-stimulated (2 and 5 min, open circles and triangles, respectively) myocytes identically induced hydrolysis of GTP on p21^{ras}. The observed ability of the lysate to induce hydrolysis of GTP reflects p21^{ras} GAP activity, because the lysate did not induce significant hydrolysis of GTP bound to a mutant p21^{ras} (G12V), which lacks intrinsic GTPase activity (Vojtek *et al.*, 1993). Thus, GAP activity was not altered by Ang II stimulation. These results suggest that the increase in GTP-bound p21^{ras} by Ang II treatment of cardiac myocytes was solely due to an enhancement of the guanine nucleotide exchange activity.

Angiotensin II stimulates protein tyrosine phosphorylation of Shc and its association with Grb2 and mSos-1

It has been shown that adapter proteins containing the SH2 domain, such as Shc and Grb2, link tyrosine-phosphorylated receptor tyrosine kinases with the guanine nucleotide exchange factor Sos to activate p21^{ras} (Pelicci *et al.*, 1992; Rozakis-Adcock *et al.*, 1992; Buday and Downward, 1993a; Egan *et al.*, 1993). Ang II rapidly (within 1 min) activates cellular tyrosine kinase activity of cardiac myocytes (Sadoshima *et al.*, 1995), and the tyrosine kinase inhibitor genistein inhibited Ang II-induced p21^{ras} activation (Figure 1D). We therefore examined whether Shc and Grb2 play a role in Ang II-induced p21^{ras} activation. The cell lysates from stimulated or unstimulated myocytes were immunoprecipitated with an anti-Shc polyclonal antibody. The immunoprecipitates were subjected to SDS–PAGE followed by immunoblotting with an anti-phosphotyrosine antibody or an anti-Shc monoclonal antibody (mAb). As shown in Figure 3A (left), within 1 min Ang II caused an increase in tyrosine phosphorylation of several proteins with approximate molecular sizes of 46, 52, 66 and 180 kDa in the anti-Shc immunoprecipitates. The bands at 46, 52 and 66 kDa in the phosphotyrosine blot (arrows, Figure 3A, left) co-migrated with the bands detected by the anti-Shc antibody

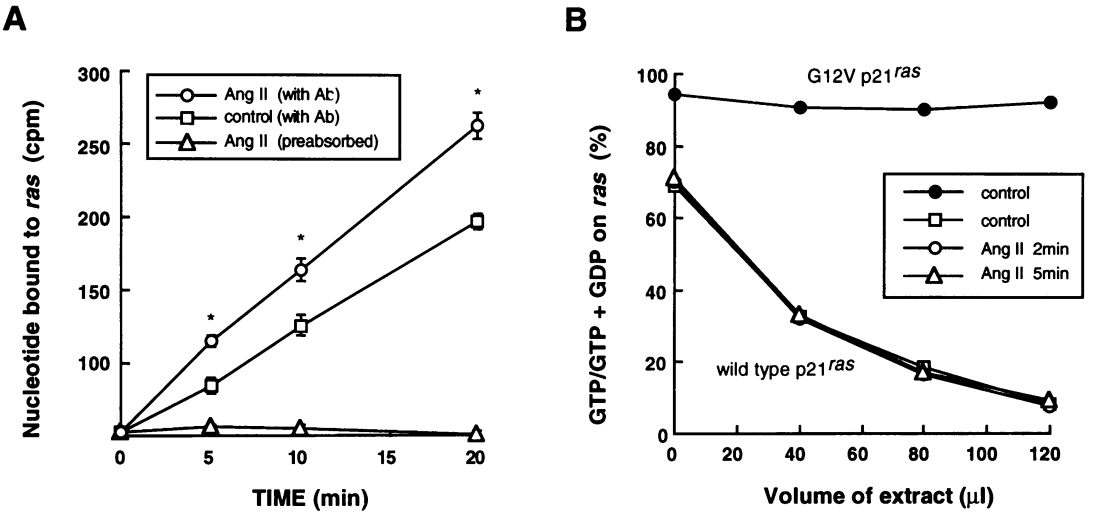

Fig. 2. Effect of Ang II on guanine nucleotide exchange activity (A) and GAP activity (B). (**A**) [α-^{32}P]GTP was added to saponin-permeabilized myocytes for the times indicated in the presence (circles) or absence (squares) of Ang II (100 nM). The cells were then lysed and p21^{ras} was immunoprecipitated. Total nucleotide binding to p21^{ras} was measured by scintillation counting. Immunoprecipitates were also prepared with a pre-absorbed p21^{ras} antibody from Ang II-stimulated permeabilized myocytes and the nucleotide binding was measured (triangles). The mean ± SE of three independent experiments is shown. Ab, antibody; $*P < 0.01$, versus control. (**B**) Cardiac myocytes were stimulated with Ang II for the times indicated and cell lysates (GAP extracts) were prepared. Recombinant wild-type p21^{ras} or mutant p21^{ras} (G12V) (500 ng) was allowed to bind [α-^{32}P]GTP (200 μCi). After the addition of 10 mM $MgCl_2$, 2 mM DTT, 1 mM GTP and 1 mM GDP, 50 ng aliquots of p21^{ras} bound to [α-^{32}P]GTP were added to varying amounts of the GAP extract, and made up to the same volume with lysis buffer. The reaction mixtures were incubated for 30 min at room temperature. The relative amounts of [α-^{32}P]GTP remaining on the p21^{ras} were plotted against the volume of lysate added. Data are averages of duplicate values and are representative of three independent experiments.

in a duplicate blot (arrows, Figure 3A, right). This suggests that they most likely correspond to Shc proteins, which are known to exist in three forms (Pelicci *et al.*, 1992), and they are tyrosine phosphorylated in response to Ang II stimulation. A band with a molecular size of ~180 kDa was strongly tyrosine phosphorylated after Ang II treatment (Figure 3A, left, open arrowhead). It did not react with the anti-Shc antibody, suggesting that p180 may be a novel Shc binding protein.

To examine whether Grb2 associates with Shc in an Ang II-dependent manner, Grb2 immunoprecipitates from stimulated or unstimulated myocytes were subjected to SDS–PAGE followed by immunoblotting with the anti-phosphotyrosine or the anti-Shc antibody. As shown in Figure 3B, increased tyrosine phosphorylation of p52 was detected in the Grb2 immunoprecipitates after stimulation with Ang II. Tyrosine phosphorylation of p46 was also detected after a longer exposure of the blot. These bands co-migrated with the bands detected by immunoblotting the Grb2 immunoprecipitates with the anti-Shc antibody (data not shown). To further confirm that Shc associates with Grb2, Shc immunoprecipitates were subjected to SDS–PAGE followed by immunoblotting with an anti-Grb2 antibody. As shown in Figure 3C, a band around 25 kDa was detected in the Shc immunoprecipitates after Ang II stimulation (lanes 2 and 3). This band co-migrated with the p25 Grb2 detected when Grb2 immunoprecipitates were immunoblotted with the anti-Grb2 antibody (Figure 3C, lane 5). These results indicate that the 46 and 52 kDa forms of Shc make a complex with Grb2 after Ang II stimulation. In addition, a tyrosine-phosphorylated protein, p180, was also detected in the Grb2 immunoprecipitates 3 min after Ang II stimulation (Figure 3B, asterisk), although at present we do not know the molecular identity of this protein.

We next examined whether Grb2 makes a complex with mSos-1, a mammalian guanine nucleotide exchange factor (Buday and Downward, 1993a; Egan *et al.*, 1993). Grb2 immunoprecipitates were subjected to immunoblotting with an anti-mSos-1 antibody after SDS–PAGE. As shown in Figure 4A, a band around 170 kDa was observed in the control state (lane 1) and its intensity increased after stimulation with Ang II (lanes 2 and 3). This 170 kDa band co-migrated with a band obtained when the mSos-1 immunoprecipitate was immunoblotted with the mSos-1 antibody (lane 5), indicating that p170 most likely corresponds to mSos-1. This p170 band was not observed when the pre-absorbed anti-Grb2 antibody was used in the immunoprecipitation (lane 4). Conversely, a faint band was observed around 25 kDa when mSos-1 immunoprecipitates obtained from control myocytes were immunoblotted with an anti-Grb2 antibody (Figure 4B, lane 1). The intensity of the 25 kDa band also increased after stimulation with Ang II (lanes 2–4). This band co-migrated with a band obtained when Grb2 immunoprecipitate was immunoblotted with the Grb2 antibody (lane 6). The p25 band was not observed when a pre-absorbed mSos-1 antibody was used for the immunoprecipitation (lane 5). We confirmed that Grb2 and mSos-1 antibodies used in the immunoprecipitations did not directly cross-react with mSos-1 and Grb2, respectively, by immunoblotting of whole-cell lysates (data not shown). These results indicate that a small fraction of Grb2 makes a complex with mSos-1 in the control state and this association seems to increase after stimulation with Ang II. This is in contrast to fibroblasts, where Grb2 and mSos-1 exist in a constitutive complex and growth factor stimulation does not alter the levels of Grb2 complexed with mSos-1 (Egan *et al.*, 1993). The increase in the levels of Grb2–mSos-1 association has been reported in T cells following T-cell receptor activation, where interaction of phosphorylated Shc with

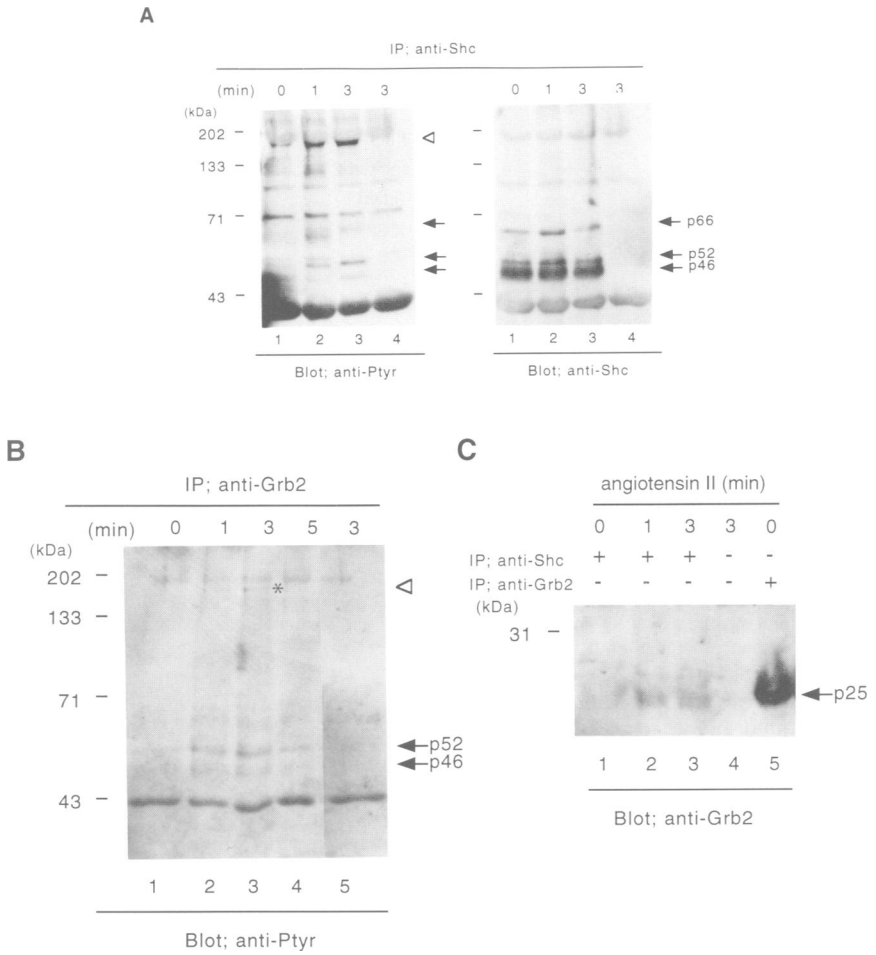

Fig. 3. Ang II-induced tyrosine phosphorylation of Shc and its association with Grb2 in cardiac myocytes. Cardiac myocytes were stimulated with 100 nM of Ang II for the indicated times. (**A**) The cell lysates were immunoprecipitated with an anti-Shc polyclonal antibody. The immunoprecipitates were analyzed by SDS–PAGE and immunoblotting, using antibodies to phosphotyrosine (left) and Shc (monoclonal, right) as indicated. In lane 4, the anti-Shc antibody was not included in the immunoprecipitation step. The positions of Shc are indicated by arrows. Sizes in kDa are indicated on the left. The white arrowhead indicates p180, which is tyrosine phosphorylated after Ang II stimulation. Ptyr, phosphotyrosine. Both blots are from the same samples electrophoresed on the same gel. (**B**) Cell lysates were immunoprecipitated with an anti-Grb2 polyclonal antibody. The immunoprecipitates were analyzed by SDS–PAGE and immunoblotted with the anti-phosphotyrosine antibody. In lane 5, the anti-Grb2 antibody was not included in the immunoprecipitation step. The positions of Shc (46 and 52 kDa forms) are indicated by arrows. The white arrowhead indicates p180, which is tyrosine phosphorylated after Ang II stimulation (lane 3, asterisk). (**C**) Cell lysates were immunoprecipitated with an anti-Grb2 polyclonal antibody or the anti-Shc polyclonal antibody as indicated. The immunoprecipitates were analyzed by SDS–PAGE and immunoblotted with an anti-Grb2 mAb. In lane 4, the anti-Shc antibody was not included in the immunoprecipitation step. The position of Grb2 (p25) is indicated by the arrow. IP, immunoprecipitation; Blot, immunoblotting.

the Grb2 SH2 domain seems to enhance Grb2/mSos-1 association (Ravichandran *et al.*, 1995).

Angiotensin II stimulates association of mSos-1 with Shc and translocates mSos-1 to the membrane fraction

We next examined whether Shc makes a complex with mSos-1 in an Ang II-dependent manner. Shc immunoprecipitates obtained from stimulated or unstimulated myocytes were subjected to immunoblotting with an anti-mSos-1 antibody after SDS–PAGE. As shown in Figure 5A, a band of apparent molecular size ~170 kDa (asterisks) was detected at time 0 and it was increased in intensity after Ang II stimulation (compare lanes 1 and 2). This band was also observed when the Shc immunoprecipitates were immunoblotted with another polyclonal mSos-1 antibody raised against the carboxy terminus of mSos-1 (data not shown). The 170 kDa band co-migrated with the band in the same gel obtained by immunoblotting the mSos-1 immunoprecipitates with the anti-mSos-1 antibody (Figure 5A, lane 3), suggesting that this band corresponds to mSos-1. Conversely, immunoblotting of the mSos-1 immunoprecipitates with the anti-Shc antibody indicated that three bands, which have apparent molecular sizes of around 46, 52 and 66 kDa, increased the intensity after stimulation with Ang II (Figure 5B, lanes 1–5). These bands co-migrated with the bands in the same gel obtained by immunoblotting the Shc immunoprecipitates with the anti-Shc antibody (lane 6). These results suggest that Shc associates with mSos-1 and the association seem to increase after Ang II stimulation.

To further confirm that mSos-1 associates with Shc, we measured $p21^{ras}$ guanine nucleotide exchange activity in the Shc immunoprecipitates *in vitro*. The guanine nucleotide exchange activity was determined as the release of [^{3}H]GDP from pre-loaded purified $p21^{ras}$, following an

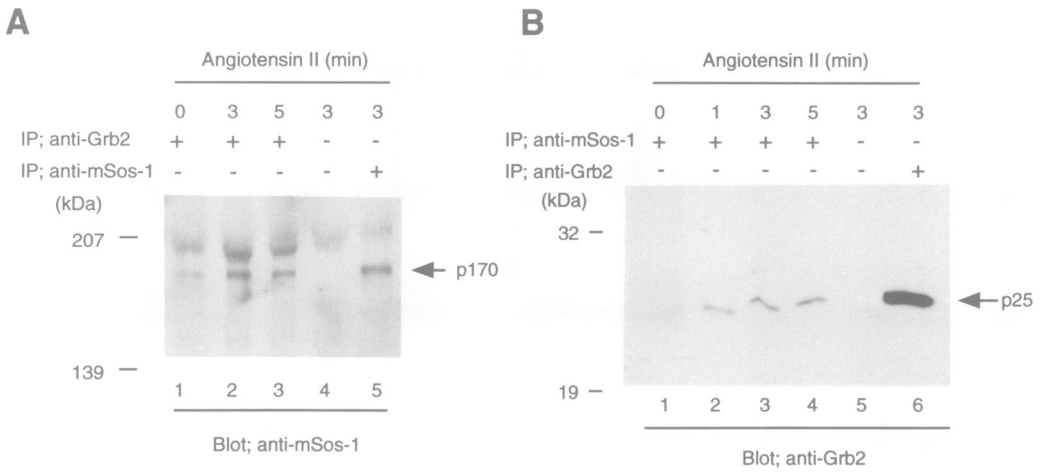

Fig. 4. Ang II-induced association of Grb2 with mSos-1. Cardiac myocytes were stimulated with 100 nM of Ang II for the indicated times. (**A**) The cell lysates were immunoprecipitated with an anti-Grb2 polyclonal antibody (lanes 1–3) or an anti-mSos-1 mAb (lane 5). In lane 4, a pre-absorbed anti-Grb2 antibody was used in the immunoprecipitation. The immunoprecipitates were analyzed by SDS–PAGE and immunoblotted with an anti-mSos-1 polyclonal antibody. The position of mSos-1 is indicated by the arrow (p170). (**B**) The cell lysates were immunoprecipitated with the anti-mSos-1 polyclonal antibody (lanes 1–4) or the anti-Grb2 polyclonal antibody (lane 6). In lane 5, a pre-absorbed anti-mSos-1 antibody was used in the immunoprecipitation. The immunoprecipitates were analyzed by SDS–PAGE and immunoblotted with an anti-Grb2 mAb. The position of Grb2 is indicated by the arrow (p25). IP, immunoprecipitation; Blot, immunoblotting.

incubation with the Shc immunoprecipitates prepared from control and Ang II-stimulated myocytes. As shown in Figure 5C, the amount of [^{3}H]GDP released from p21^{ras} increased 2.3-fold at 3 min after Ang II stimulation. The [^{3}H]GDP release was very low in immunoprecipitates prepared with a non-immune serum (hatched bar). This indicates that the guanine nucleotide exchange activity, most likely from mSos-1, is present in the Shc immune complex and increases significantly after Ang II stimulation. [^{3}H]GDP release in the control state (white bar) was significantly higher than that observed in immunoprecipitates prepared with a non-immune serum, suggesting the existence of a basal association between Shc and guanine nucleotide exchange activity. These results corroborate the results obtained by co-immunoprecipitation of mSos-1 and Shc shown in Figure 5A and B.

Because p21^{ras} is localized at the plasma membrane, we examined whether Ang II treatment changes the intracellular localization of mSos-1 and thereby modulates its access to its substrate. Membrane (particulate) and cytosolic (soluble) fractions were prepared from control and Ang II-stimulated myocytes. The mSos-1 protein was then immunoblotted from both cellular components. Fractionation of the control myocytes indicated that mSos-1 protein was predominantly localized in the cytosolic fraction, although a small amount was detected in the particulate fraction (Figure 5D, time 0). In response to Ang II treatment, a portion of mSos-1 translocated to the particulate fraction in a time-dependent manner. While we do not know to which component in the particulate fraction mSos-1 actually translocates, it is likely that Ang II stimulation of myocytes results in the mSos-1 protein moving to the cell membrane by its association with Grb2 and Shc. A similar translocation has been observed after receptor tyrosine kinase stimulation (Buday and Downward, 1993a).

Shc associates with Fyn, a member of the Src family tyrosine kinases

Shc is known to be tyrosine phosphorylated by the Src family kinases (McGlade *et al.*, 1992). Moreover, it has recently been shown that the proline-rich amino acid sequences of Shc interact with the SH3 domain of the Src family kinases *in vitro* (Weng *et al.*, 1994). Cardiac myocytes express the Src family tyrosine kinases, such as Src and Fyn (our unpublished observation). We therefore examined whether such interaction between Shc and the Src family kinases, such as Fyn, occurs in cardiac myocytes *in vitro*. We focused on Fyn because our anti-Fyn mAb (clone 15) was reported to be non-cross-reactive with other Src family tyrosine kinases, while anti-Src antibody (clone 327) was cross-reactive with other Src family tyrosine kinases as well. In addition, three-dimensional structures of Fyn and Src SH3 domains are very similar (Yu *et al.*, 1992). Lysates prepared from myocytes were incubated with an agarose-conjugated glutathione-S transferase (GST)-Fyn SH3 domain (amino acids 85–139). The bound proteins were eluted and immunoblotted with the anti-Shc antibody. As shown in Figure 6A, an association of GST-Fyn SH3 with the 52 and 66 kDa forms of Shc was observed in both control (lane 1) and Ang II-stimulated (lane 2) myocytes. The association with Shc was also detected in GST-Fyn containing both SH2 and SH3 domains (amino acids 85–247, lane 4), but not in GST-Fyn containing only the SH2 domain (amino acids 145–247, lane 3) or GST alone (lane 5). The association with Shc was not observed when the GST-Grb2 SH3 domain was used (data not shown). These results suggest that the association was specific to the Fyn SH3 domain and raises the possibility that a fraction of Shc may constitutively associate with Fyn. To examine whether Shc and Fyn physically interact *in vivo*, the Shc immunoprecipitates were immunoblotted with a specific anti-Fyn antibody after SDS–PAGE. We found that a protein around 60 kDa was co-immunoprecipitated with Shc (Figure 6B, lane 1) and this was co-migrated with the Fyn immunoprecipitates (lane 2). Reprobing the blot with the anti-Shc antibody confirmed that this band was not Shc itself (not shown). These results indicate that a small fraction of Fyn may associate with Shc *in vivo*.

We next examined whether Ang II stimulates tyrosine

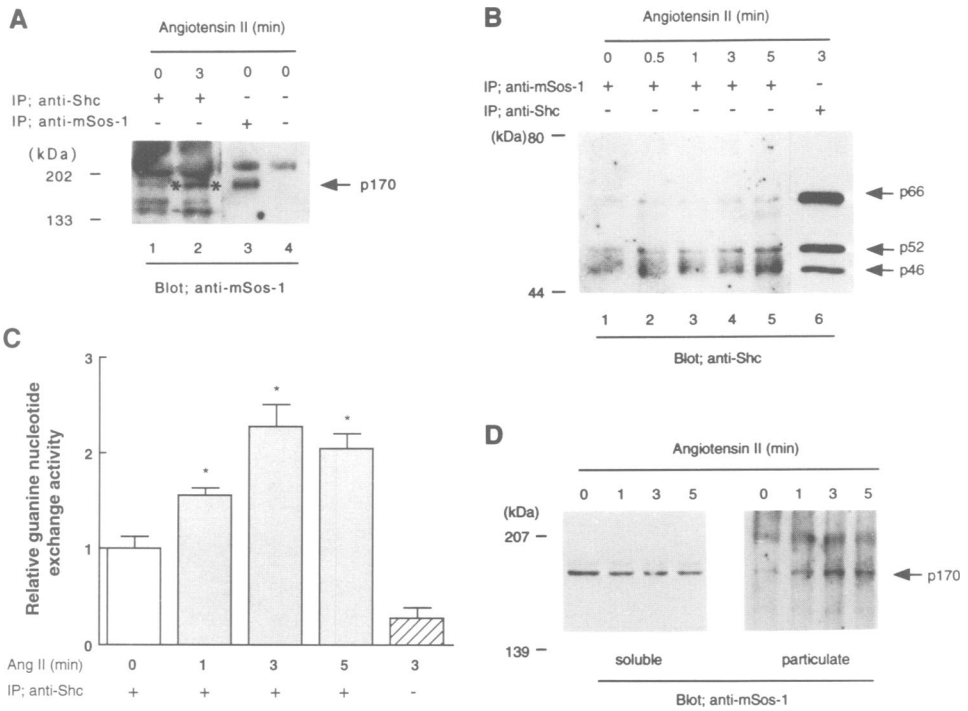

Fig. 5. Ang II induces association of mSos-1 with Shc and translocates mSos-1 to the particulate fraction. Cardiac myocytes were stimulated with 100 nM of Ang II for the indicated times. (**A**) Cell lysates were immunoprecipitated with an anti-Shc polyclonal antibody (lanes 1 and 2) or an anti-mSos-1 mAb (lane 3). In lane 4, anti-mSos-1 antibody was not included in the immunoprecipitation step. The immunoprecipitates were analyzed by SDS–PAGE and immunoblotted with an anti-mSos-1 polyclonal antibody. The position of mSos-1 is indicated by asterisks and the arrow (p170). Note that lanes 1, 2 and 3, 4 are from the same gel. Densitometric analysis indicates that mSos-1 associated with Shc in lane 2 was 26% of the total mSos-1 immunoprecipitated in lane 3. (**B**) Cell lysates were immunoprecipitated with the anti-mSos1 mAb (lanes 1–5) or the anti-Shc polyclonal antibody (lane 6). The immunoprecipitates were then immunoblotted with an anti-Shc mAb. The positions of Shc are indicated by arrows (p46, p52, p66). IP, immunoprecipitation; Blot, immunoblotting. Note that lanes 1–5 and 6 are from the same gel, but the latter was exposed for a shorter time period. The density of the p52-Shc band in lane 5 was 5% of that in lane 6, when compared in the same autoradiograph. (**C**) Guanine nucleotide exchange activity in Shc immunoprecipitates. The Shc immunoprecipitates were prepared as in (A). Recombinant wild-type $p21^{ras}$ pre-loaded with [^{3}H]GDP was incubated with the Shc immunoprecipitates, control immunoprecipitates (with non-immune serum) or buffer alone for 10 min at 30°C. After fixation of $p21^{ras}$ on a nitrocellulose membrane, the remaining [^{3}H]GDP on $p21^{ras}$ was determined by scintillation counting. The guanine nucleotide exchange activity was defined as [^{3}H]GDP released during the 10 min incubation, which was obtained by subtracting [^{3}H]GDP remaining on $p21^{ras}$ after incubation with the immunoprecipitates from that after incubation with buffer alone. The amount of released [^{3}H]GDP in the Shc immunoprecipitates from unstimulated myocytes was designated as 1.0. The results are the mean ± SE obtained from four experiments. $*P < 0.05$ versus control (time zero). (**D**) Effect of Ang II stimulation on subcellular distribution of mSos-1. After stimulation of myocytes, subcellular fractionation was performed. The samples containing equal amounts of total protein (40 μg) were subjected to SDS–PAGE on a 7.0% gel and were then immunoblotted with the anti-mSos-1 polyclonal antibody. The position of mSos-1 is indicated by the arrow. The cytosolic and particulate fractions obtained from the same sample are shown. The filter of the soluble fraction was exposed for a shorter time than that of the particulate fraction because of the relative abundance of mSos-1 in the soluble fraction. The result shown is representative of five independent experiments.

kinase activity of Fyn. Fyn was immunoprecipitated and the tyrosine kinase activity of Fyn was determined by the immune complex kinase assay, using Raytide as a substrate, as well as an autophosphorylation assay. As shown in Figure 6C, an increase in tyrosine kinase activity toward Raytide and autophosphorylation of Fyn were both observed as early as 1 min, and its level reached an apparent peak ~3 min after stimulation with Ang II. The observed 2- to 3-fold increase in Fyn kinase activity by Ang II was similar to that by PDGF stimulation in NIH 3T3 fibroblasts (Kypta *et al.*, 1990). A similar result was obtained when the immunoprecipitation was performed using another anti-Fyn antibody (not shown). The increase in autophosphorylation was observed even if the gel was treated with KOH to eliminate serine/threonine phosphorylation (data not shown), suggesting that autophosphorylation was due to an increase in the tyrosine kinase activity of Fyn. The Ang II-induced increase in the Fyn kinase activity toward Raytide was completely suppressed in the presence of genistein. These results indicate that stimulation with Ang II causes an increase in tyrosine kinase activity of Fyn. It remains to be determined whether Ang II also increases the kinase activity of other members of the Src family.

The Src family tyrosine kinases but not PKC play an essential role in Ang II-induced activation of $p21^{ras}$

To examine the role of the Src family kinases in Ang II-induced activation of $p21^{ras}$, guanine nucleotide exchange activity was measured in the presence of the Src-Pro peptide, which contains the sequence identified as a specific Src family kinase (Fyn, Lyn and Src) SH3 ligand by the phage display selection (Rickles *et al.*, 1994), or an unrelated control peptide in the permeabilized cell assay. The Src-pro peptide significantly suppressed the Ang II-induced increase in guanine nucleotide exchange activity (Figure 6D, closed squares), while the control peptide did not (closed triangles). The Src-Pro peptide specifically inhibits binding of p52 Shc to GST-Fyn

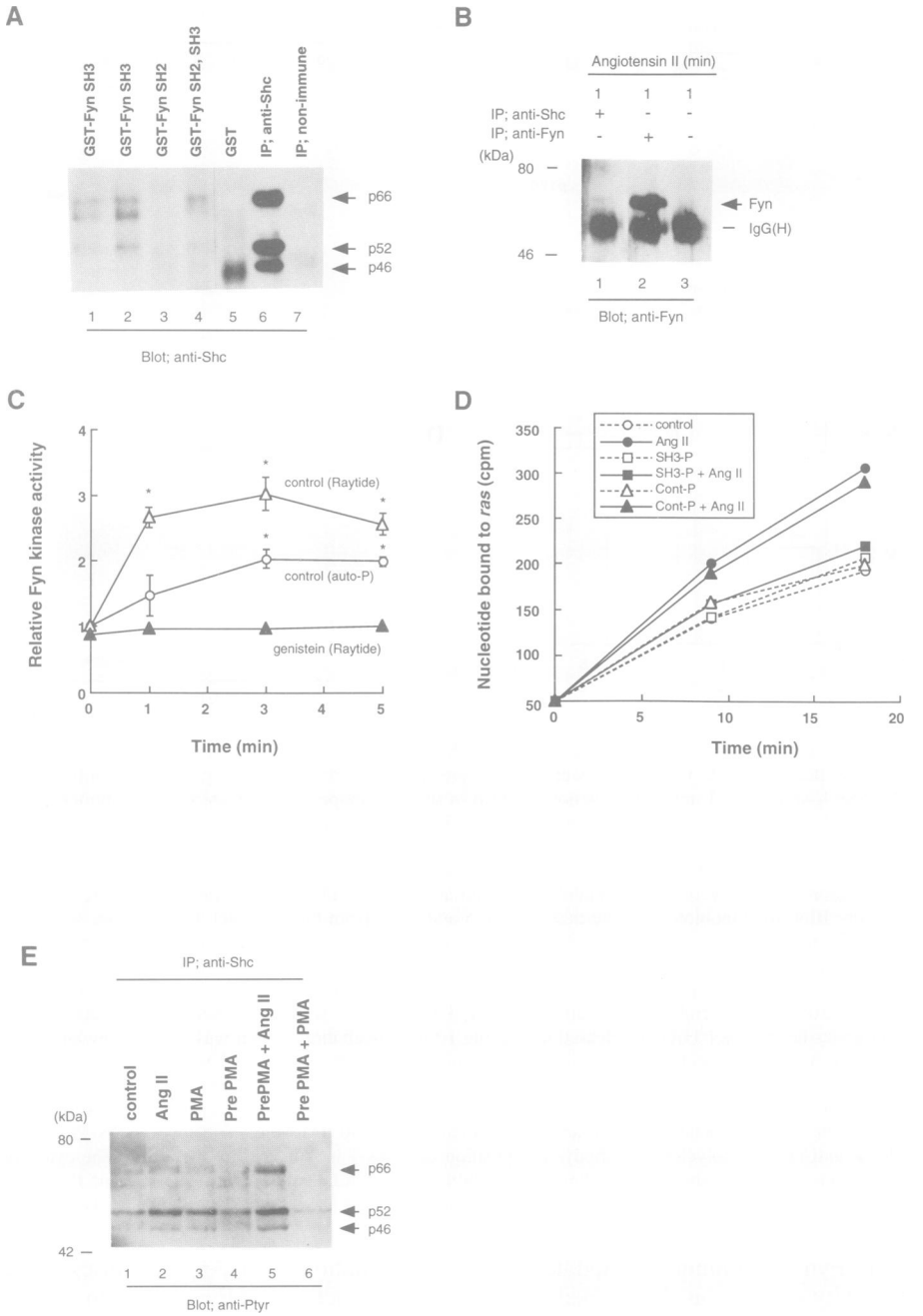

Fig. 6. (**A**) Association of Shc with the SH3 domain of Fyn *in vitro*. Myocytes were lysed without stimulation (lanes 1, 3–7). Some myocytes were lysed after stimulation with Ang II (100 nM) for 3 min (lane 2). The cell lysates were incubated with glutathione–agarose bound with GST-Fyn SH3 (lanes 1 and 2), GST-Fyn SH2 (lane 3), GST-Fyn SH2, SH3 (lane 4) and GST alone (lane 5). The bound proteins were eluted and subjected to SDS–PAGE, along with an immunoprecipitate using an anti-Shc polyclonal antibody (lane 6), and a sample immunoprecipitated with non-immune serum (lane 7), and immunoblotted with the anti-Shc polyclonal antibody. The positions of Shc are indicated by arrows. (**B**) Interaction between Shc and Fyn *in vivo*. Shc and Fyn were immunoprecipitated with specific antibodies. In lane 3, the anti-Fyn antibody was pre-absorbed with excess antigen peptide. Immunoprecipitates were immunoblotted with specific anti-Fyn polyclonal antibody. The results shown are representative of four and two independent experiments in (A) and (B), respectively. (**C**) Ang II activates tyrosine kinase activity of Fyn. Myocytes were stimulated with Ang II (100 nM) in the presence or absence (control) of genistein (30 μM) for the indicated times. Cell lysates were immunoprecipitated with an anti-Fyn polyclonal antibody and the immunoprecipitates were then subjected to kinase assay using Raytide as a substrate (triangles) or autophosphorylation (auto-P, circles). The results of the scintillation counting (for Raytide) or densitometric analysis of the autoradiographs (for auto-P) are shown. The level of Fyn kinase activity at time zero was designated as one. Immunoprecipitates with a pre-absorbed anti-Fyn antibody showed little autophosphorylation (not shown). Results are the mean ± SE obtained from three independent experiments. $*P < 0.05$ versus time zero. (**D**) Guanine nucleotide exchange activity was determined by a permeabilized cell assay. Myocytes were pre-treated with the permeabilization buffer containing the Src-Pro peptide (40 μM) or unrelated control peptide (40 μM) for 5 min and then treated with Ang II (100 nM) and [α-^{32}P]GTP for the times indicated. p21^{ras} was immunoprecipitated and the bound [α-^{32}P]GTP was counted. Results are representative of two separate experiments performed in duplicate. SH3-P, Src-Pro peptide; Cont-P, control peptide. (**E**) Effects of PKC downregulation on Ang II-induced tyrosine phosphorylation of Shc. Myocytes were pre-treated with PMA (PrePMA, 1 μM) for 48 h and then stimulated with Ang II (100 nM) or PMA (1 μM) for 5 min. The cell lysates were immunoprecipitated by a polyclonal anti-Shc antibody. The immunoprecipitates were analyzed by SDS–PAGE and immunoblotted with an anti-phosphotyrosine antibody. The positions of Shc are indicated by the arrows. IP, immunoprecipitation; Blot, immunoblotting; Ptyr, anti-phosphotyrosine.

SH3 *in vitro* (data not shown). It also inhibited co-immunoprecipitation of Fyn with Shc in permeabilized myocytes (data not shown). Furthermore, inclusion of the PKC pseudosubstrate peptide, which we have shown to inhibit fully Ang II-induced activation of PKC in the permeabilized cell assay (Sadoshima and Izumo, 1993c), failed to inhibit the Ang II-induced increase in guanine nucleotide exchange activity (data not shown). These results suggest that Src family tyrosine kinases, but not PKC, may play an essential role in Ang II-induced activation of $p21^{ras}$. In order to confirm that PKC is not required for Ang II-induced Shc phosphorylation, we examined the phosphotyrosine contents of Shc by immunoprecipitating Shc followed by anti-phosphotyrosine immunoblotting (Figure 6E). While direct stimulation of PKC by PMA could cause a moderate increase in tyrosine phosphorylation of Shc (lane 3), downregulation of PKC by a prolonged treatment with PMA did not affect Ang II-induced tyrosine phosphorylation of Shc (lane 5). Functional downregulation of PKC after PMA pre-treatment is confirmed by the lack of Shc tyrosine phosphorylation when PMA pre-treated myocytes were re-challenged by PMA (lane 6). Therefore, phorbol-sensitive forms of PKC are not required for Ang II-induced $p21^{ras}$ activation.

Discussion

Recent biochemical and genetic studies on signal transduction of tyrosine kinase receptors have demonstrated that $p21^{ras}$ serves as a nodal point for transmitting signals from receptor tyrosine kinases to the downstream serine/threonine kinase cascades (McCormick, 1994; Burgering and Bos, 1995; Hunter, 1995). A number of cytokine receptors seem to utilize a similar pathway for $p21^{ras}$ activation via stimulation of the receptor-associated Src family tyrosine kinases in response to ligand-induced receptor dimerization (Heldin, 1995). In contrast, the mechanisms of $p21^{ras}$ activation by heterotrimeric G protein-coupled receptors have remained elusive, as no member of the serpentine receptor family has been shown to have intrinsic kinase activity or is known to have receptor-associated tyrosine kinases. Evolutionarily, the mechanism of $p21^{ras}$ activation by G protein-coupled receptors should be more ancient than activation by tyrosine kinases. Yeasts, for example, do not have tyrosine kinases, but have serpentine receptors, Ras and MAP kinase cascades (Herskowitz, 1995). In fission yeast, the mating pheromone binds to the G protein-coupled receptor, which results in activation of ras1 via STE6, a guanine nucleotide exchange factor for ras1. The biochemical link between the receptor and STE6 is unknown, although the G_α subunit seems to be required for ras1 activation (Herskowitz, 1995).

In the present study, we found that $p21^{ras}$ is activated by Ang II in cardiac myocytes. The neonatal rat cardiac myocytes in our culture conditions are unable to pass through G1/S transition even after strong mitogen stimulation (our unpublished results). This indicates that $p21^{ras}$ may have functions unrelated to cell proliferation in cardiac myocytes. In this regard, it is interesting to note that expression of a dominant negative $p21^{ras}$ (N17) in cardiac myocytes was reported to result in a 'global' inhibition of gene expression (Abdellatif *et al.*, 1994).

Activation of guanine nucleotide exchange activity by receptor tyrosine kinases, as well as non-receptor-type tyrosine kinases, in response to growth factors and cytokines is mediated by the association of SH2/SH3-containing adapter proteins, such as Shc and Grb2, with the guanine nucleotide exchange factor mSos-1 (Buday and Downward, 1993a,b; Egan *et al.*, 1993; Medema *et al.*, 1993). Ang II causes tyrosine phosphorylation of Shc and its association with Grb2 and mSos-1, indicating that the agonist for a pertussis toxin-insensitive G protein-coupled receptor can utilize a mechanism for $p21^{ras}$ activation similar to that used by receptor-type or non-receptor-type tyrosine kinases. The G_i-coupled receptor-mediated $p21^{ras}$ activation by thrombin and lysophosphatidic acid in Rat-1 cells is sensitive to genistein (Hordijk *et al.*, 1994), whereas that by a muscarinic type agonist in COS cells is not (Winitz *et al.*, 1993). It is likely that the G protein agonist-induced $p21^{ras}$ activation is mediated by multiple mechanisms depending on the type of receptor, the G protein isoforms expressed and cell backgrounds.

In cardiac myocytes, Ang II receptors (AT_{1A} and AT_{1B} receptors) couple to the G_q class of G proteins (Gutowski *et al.*, 1991). Recently, it has been suggested that the $\beta\gamma$ subunit of G proteins ($G_{\beta\gamma}$) mediates activation of $p21^{ras}$ by G_i-coupled receptor agonists, but not by G_q-coupled receptor agonists, such as α_1-adrenergic agonists (Koch *et al.*, 1994). Because $G_{\beta\gamma}$ contains a region encompassing a pleckstrin homology (PH) domain, $p21^{ras}$ activation by G_i protein-coupled receptors may involve a complex formation between $G_{\beta\gamma}$ and proteins having the PH domain, such as mSos-1, Ras-GRF, Ras-GAP and some tyrosine kinases (Koch *et al.*, 1994; Langhans-Rajasekaran *et al.*, 1995; Shou *et al.*, 1995). It remains to be determined whether $G_{\beta\gamma}$ plays a role in Ang II-induced $p21^{ras}$ activation. It is interesting to note that the MAP kinase activation by M_1 muscarinic receptor (which is coupled to G_q) transfected in COS-7 cells is mediated by $\beta\gamma$ subunits acting on a $p21^{ras}$-dependent pathway (Crespo *et al.*, 1994).

Recently, it has been reported that the AT_{1A} receptor is phosphorylated on its serine and tyrosine residues upon stimulation with Ang II (Kai *et al.*, 1994; Paxton *et al.*, 1994). It is possible that the phosphotyrosine residues may provide the binding sites for Grb2 or Shc. However, the cytoplasmic domains of the rat AT_{1A} and AT_{1B} receptors do not possess the consensus recognition motif of Grb2-SH2 (phosphoTyr-Ile/Val-Asn-X), Shc-SH2 (phosphoTyr-hydrophobic/Glu-X-Ile/Leu/Met or Asn-Pro-X-phosphoTyr) (Songyang *et al.*, 1994), or Shc-phosphotyrosine binding (PTB) domain (Asn-Pro-X-phosphoTyr) (van der Geer *et al.*, 1995). Moreover, the significant increase in tyrosine phosphorylation of the AT_{1A} receptor by Ang II was observed only after 10 min (Kai *et al.*, 1994). Therefore, it is highly unlikely that tyrosine phosphorylation of the AT_{1A} receptor accounts for the Ang II-induced $p21^{ras}$ activation. Recent evidence suggests that the interaction of Shc or Grb2 with receptor tyrosine kinases is not required for activation of $p21^{ras}$, but the formation of a complex among Shc, Grb2 and mSos-1 seems critical in nerve growth factor-induced $p21^{ras}$ activation (Basu *et al.*, 1994). We have shown that Grb2 and mSos-1 associate with tyrosine-phosphorylated Shc, and a significant increase in guanine nucleotide exchange activity was

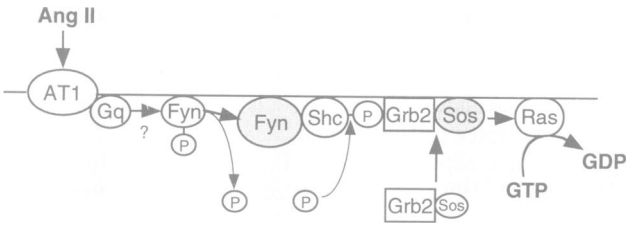

Fig. 7. Proposed mechanisms of the G_q agonist-induced $p21^{ras}$ activation. Ang II binding to the AT_1 receptor activates Src family tyrosine kinases such as Fyn. The mechanism of Ang II-induced Fyn activation remains to be elucidated. Shc physically interacts with Fyn. Shc is tyrosine phosphorylated and serves as an independent docking site for the Grb2–mSos-1 complex. Translocation of mSos-1 to the membrane fraction causes activation of $p21^{ras}$. The shaded forms Fyn and Sos indicate active forms induced by translocation or other mechanisms. P, phosphate.

detected in the Shc immunoprecipitates after stimulation with Ang II. This suggests that the tyrosine-phosphorylated Shc may work as an independent docking site for Grb2–mSos-1 complex in Ang II-stimulated myocytes.

It has been shown that Shc is tyrosine phosphorylated in fibroblasts by G protein-coupled receptor agonists (Cazaubon *et al.*, 1994; Ohmichi *et al.*, 1994; Schorb *et al.*, 1994). However, it is not known how stimulation of the G protein-coupled receptors leads to tyrosine phosphorylation of Shc. The direct or indirect association of the receptors with non-receptor-type tyrosine kinases has been shown for cytokine receptors and lymphocyte antigen receptors (Weiss and Littman, 1994; Taniguchi, 1995). In cytokine receptors, the JAK family tyrosine kinases directly associate with the cytoplasmic domain of the receptors (Taniguchi, 1995). Members of two classes of non-receptor tyrosine kinases, Src family and Syk/ZAP 70, have been shown to interact with T- and B-cell antigen receptors as well as cytokine receptors in lymphocytes (Weiss and Littman, 1994; Taniguchi, 1995). This led us to examine the role of the Src family tyrosine kinases in Ang II-induced tyrosine phosphorylation of Shc. Our results suggest that Fyn, and probably other members of the Src family tyrosine kinases, may mediate, at least in part, Ang II-induced $p21^{ras}$ activation. Future studies should include the determination of whether Ang II can activate $p21^{ras}$ in cardiac myocytes from *Fyn*$^-$ mice (Stein *et al.*, 1992). Because of potential functional redundancies among the Src family kinases, it may be necessary to use the cells that are null for multiple members of the Src kinase family genes.

At present, we do not know how Fyn interacts with the Ang II receptor and increases its tyrosine kinase activity. Because dephosphorylation of the phosphotyrosine residue at the carboxy terminus is one of the mechanisms of activation of Fyn and other members of the Src kinase family (Cooper and Howell, 1993), it is conceivable that tyrosine phosphatases are involved in Ang II-induced activation of Fyn. Alternatively, there may be an as yet unidentified, direct link between G_q protein and the Src family tyrosine kinases (Figure 7). The cytoplasmic domain of the AT_1 receptor has been shown to be a good substrate for the Src family tyrosine kinases *in vitro* (Paxton *et al.*, 1994), raising a possibility that the Src family tyrosine kinases may physically interact with the AT_1 receptor. It remains to be determined whether the AT_1 receptor is a physiological substrate for the Src family kinases *in vivo*.

It has recently been shown that Ang II causes tyrosine phosphorylation of JAK2 and its association with type 1 Ang II receptor in vascular smooth muscle cells (Marrero *et al.*, 1995). However, it remains to be elucidated whether JAK2 plays a role in Ang II-induced $p21^{ras}$ activation, because Ang II did not cause a significant increase in tyrosine phosphorylation of JAK 2 in cardiac myocytes (our unpublished observation). Very recently, Lev *et al.* (1995) reported that bradykinin, a G_q agonist, activates PYK2, a brain-specific Ca^{2+}-sensitive tyrosine kinase, in PC 12 cells and that tyrosine-phosphorylated PYK2 can directly and indirectly recruit Grb2, suggesting that PYK2 may mediate G_q agonist-induced $p21^{ras}$ activation. Involvement of similar tyrosine kinases is, however, less likely in our system, because PYK2 is not expressed in cardiac myocytes and Ca^{2+} ionophore was not sufficient to activate $p21^{ras}$.

In summary, we have demonstrated that Ang II rapidly activates $p21^{ras}$ in cardiac myocytes by tyrosine phosphorylation of Shc, causing its association with Grb2 and mSos-1, with the subsequent activation of guanine nucleotide exchange activity. While this paper was under review, it has been suggested that $p21^{ras}$ activation by pertussis toxin-sensitive G protein agonists also critically depends upon tyrosine phosphorylation of Shc (van Biesen *et al.*, 1995). Thus, the signaling pathway for $p21^{ras}$ activation by G protein-coupled receptors seems to converge with that by tyrosine kinase receptors at the level of tyrosine phosphorylation of the adapter protein Shc.

Materials and methods

Cell culture

Primary cultures of cardiac ventricular myocytes from 1-day-old Wistar rats were prepared and plated on gelatin-coated 60 or 100 mm dishes as described previously (Sadoshima *et al.*, 1992). After cell attachment, the culture medium was changed to a defined serum-free medium for 48 h before Ang II treatment.

Metabolic labeling, immunoprecipitation and determination of guanine nucleotides bound to $p21^{ras}$

Myocytes were labeled with [^{35}S]methionine (NEN) at 0.1 mCi/ml in methionine-free DMEM (Gibco) for 12 h. Myocytes were lysed with lysis buffer A [50 mM HEPES (pH 7.4), 1% Triton X-100, 50 mM NaCl, 20 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 10 mM benzamidine, 1 mM AEBSF, 0.1 mM leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor and 1 mM Na orthovanadate]. Nuclei were removed by centrifugation at 15 000 *g* for 10 min and 500 mM NaCl, 0.5% Na deoxycholate and 0.05% SDS added to the lysate. Immunoprecipitation was for 50 min using anti-$p21^{ras}$ antibody precoupled to protein A–agarose (Y13-259). Immunoprecipitates were washed with 8×1 ml of a wash buffer containing 50 mM HEPES (pH 7.4), 500 mM NaCl, 5 mM $MgCl_2$ and 0.005% SDS. Immunoprecipitates were subjected to SDS–PAGE, followed by autoradiography. Analysis of $p21^{ras}$-associated guanine nucleotides was performed by an immunoprecipitation assay as described previously (Downward *et al.*, 1990). Myocytes cultured in 60 mm dishes were labeled with [^{32}P]orthophosphate (NEN) at 0.1 mCi/ml in phosphate-free DMEM (Gibco) for 8 h. Cell lysis and immunoprecipitation of $p21^{ras}$ were performed as above. Nucleotide was eluted with 2 mM EDTA, 2 mM dithiothreitol (DTT), 0.2% SDS, 0.5 mM GTP and 0.5 mM GDP at 68°C for 20 min. Separation of eluted nucleotide was on PEI–cellulose plates (Sigma, T6765) run in 1.2 M ammonium formate and 0.8 M HCl. The positions of GTP and GDP standards were determined under UV light or by migration of [α-^{32}P]GTP and [^{3}H]GDP, respectively. Radiolabeled nucleotides were quantitated by densitometry.

Measurement of guanine nucleotide exchange activity in permeabilized myocytes

Guanine nucleotide exchange activity was measured by a modification of the permeabilized cell assay as described previously (Downward *et al.*, 1990). Cell permeabilization was performed by placing cells in 1.0 ml of a permeabilization buffer [150 mM KCl, 37.5 mM NaCl, 6.25 mM $MgCl_2$, 0.8 mM EGTA, 1 mM $CaCl_2$, 1.25 mM ATP, 12.5 mM PIPES (pH 7.4), 0.02% saponin] at 37°C. In some experiments, the Src-Pro peptide (KGGRSLRPLPPLPPPG) or control peptide (KGELRLR-NYYYDVV) (Weng *et al.*, 1994) was included in the permeabilization buffer. After 5 min, 5 μCi of [α-^{32}P]GTP (3000 Ci/mmol) were added along with Ang II. $p21^{ras}$ was immunoprecipitated as above and radioactivity was counted.

Measurement of GAP activity

The GAP activity was measured as described previously (Downward *et al.*, 1990). Lysates were made by disrupting cells in 0.5 ml of 10 mM PIPES buffer (pH 7.2), containing 120 mM KCl, 30 mM NaCl, 5 mM free Mg^{2+}, 100 nM free Ca^{2+}, 1% Triton X-100, 10% glycerol and 100 nM okadaic acid. The lysate was centrifuged at 300 000 *g* for 10 min and the supernatants assayed for GAP activity. Five hundred nanograms of recombinant wild-type $p21^{ras}$ (UBI) or mutant $p21^{ras}$ (G12V, UBI, 500 ng) were allowed to bind 200 μCi of [α-^{32}P]GTP in the presence of 5 mM EDTA at 37°C for 5 min, then 10 mM $MgCl_2$, 1 mM GTP and 1 mM GDP were added. Fifty nanogram aliquots of $p21^{ras}$ bound to [α-^{32}P]GTP were added to varying amounts of GAP extracts and made up to the same volume with the lysis buffer. The mixtures were incubated for 5 min at 37°C, the reaction stopped by the addition of 10 vols of cold lysis buffer containing 500 mM NaCl, and $p21^{ras}$ immunoprecipitated. The proportion of [α-^{32}P]GTP to total labeled nucleotides on $p21^{ras}$ was calculated following separation of GTP from GDP by TLC.

Immunoprecipitation and immunoblot analysis

Cell lysates were prepared with lysis buffer B [50 mM HEPES (pH 7.0), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM NaF, 10 mM Na pyrophosphate, 1 mM AEBSF, 10 μg/ml aprotinin, 0.1 mM leupeptin and 0.1% SDS]. Equal amounts (900 μg) of lysates were pre-cleared with Pansorbin (Calbiochem) and then incubated with an anti-Shc polyclonal antibody (UBI, 4 μg), an anti-Grb2 polyclonal antibody (Santa Cruz, 1.5 μg), or an anti-mSos-1 mAb (Transduction Laboratory, 1 μg) at 4°C for 12 h. Protein A–Sepharose (Pharmacia) was then added. For immunoprecipitations with the mAb, an anti-mouse IgG rabbit antibody (2 μg, Jackson) was added before the addition of protein A. The Sepharose beads were washed five times with the lysis buffer. Proteins were subjected to SDS–PAGE and transferred to immobilon-P membranes (Millipore). Western blot analysis was performed as described previously (Sadoshima *et al.*, 1995). A peroxidase-conjugated anti-phosphotyrosine recombinant antibody at a dilution of 1:2500 (RC20H, Transduction Laboratory), an anti-Shc mAb at 1 μg/ml (Transduction Laboratory), an anti-Grb2 mAb at 2.5 μg/ml (MBL), an anti-mSos-1 polyclonal antibody at 1 μg/ml (Santa Cruz) or anti-Fyn polyclonal antibody at 1 μg/ml (Santa Cruz) was used as the primary antibody. A peroxidase-conjugated anti-mouse IgG antibody (Jackson) and a peroxidase-conjugated protein G (Zymed) were used as secondary antibodies for immunoblotting with the anti-Shc antibody and the anti-Grb2 antibody, respectively. The signal was visualized with the ECL system (Amersham) or with [^{125}I]protein A (ICN) for the anti-mSos-1 antibody.

In vitro guanine nucleotide exchange assay

The guanine nucleotide exchange activity in the Shc immunoprecipitates was determined by an *in vitro* guanine nucleotide exchange assay. Recombinant wild-type $p21^{ras}$ (UBI, 500 ng) was pre-loaded with 1 μM [^{3}H]GDP (10 μCi/ml) by a 20 min incubation at 37°C in 20 mM HEPES (pH 7.5), 5 mM EDTA and 5 μg/ml bovine serum albumin. The reaction was stopped on ice by addition of $MgCl_2$ to 25 mM final concentration. The $p21^{ras}$–[^{3}H]GDP complex was incubated with the Shc immunoprecipitates for 10 min at 30°C in the presence of unlabeled 150 mM GDP, 1.5 mM GTP and 20 mM $MgCl_2$. The reaction was stopped by filtering the reaction mixture through nitrocellulose filters (Biorad, 0.45 μm) using the dot blot apparatus (Biorad). The filters were washed with ice-cold buffer containing 20 mM sodium phosphate (pH 6.5), 2.5 mM $MgCl_2$, 1 mM DTT and 100 mM NaCl. The filters were placed in scintillation fluid and counted for [^{3}H]GDP that remained on $p21^{ras}$.

Subcellular fractionation

After stimulation with Ang II, myocytes (10 cm dishes) were washed with ice-cold PBS, scraped from the dish in 0.5 ml of lysis buffer C [25 mM Tris–HCl (pH 7.4), 5 mM EGTA, 2 mM EDTA, 100 mM NaF, 5 mM DTT, 0.2 mM Na orthovanadate, 0.1 mM pepstatin, 1 mM AEBSF, 10 μg/ml aprotinin and 0.1 mM leupeptin] and immediately frozen in liquid nitrogen. The myocytes were disrupted by three cycles of a rapid freeze and thaw. The lysate was centrifuged at 100 000 *g* at 4°C for 1 h. The supernatant was used as the cytosolic fraction. The pellet was further extracted with 0.5 ml of lysis buffer B plus 1% SDS and recentrifuged. The detergent extract was used as the particulate fraction.

Affinity binding assay and kinase assay for Fyn

Myocytes were lysed with lysis buffer B. Aliquots of lysates containing equal amounts of protein (750 μg) were incubated with 10 μg of glutathione agarose-conjugated GST, GST-Fyn SH3 (amino acids 85–139 of human Fyn), GST-Fyn SH2 (amino acids 145–247), GST-Fyn SH2 and SH3 (amino acids 85–247) or GST-Grb2 SH3 (amino acids 156–199 of mouse Grb2) (Santa Cruz) for 5 h at 4°C. The pellets were washed four times with the lysis buffer, and the binding proteins eluted with Laemmli's buffer at 100°C for 10 min. The samples were subjected to SDS–PAGE on a 10% gel and then immunoblotted with an anti-Shc mAb. The tyrosine kinase activity of Fyn was determined in the immune complex by a kinase assay using Raytide (Oncogene Science) as a substrate or an autophosphorylation assay. Myocytes were lysed with lysis buffer D [150 mM NaCl, 10 mM Na phosphate (pH 7.0), 0.1% SDS, 1.0% NP-40, 1.0% Na deoxycholate, 2 mM EDTA, 50 mM NaF, 1 mM Na orthovanadate, 1 mM AEBSF, 0.1 mM leupeptin, 10 μg/ml aprotinin and 10 μg/ml soybean trypsin inhibitor]. Equal aliquots (750 μg) of the lysates were incubated with an anti-Fyn polyclonal (Santa Cruz, 1.5 μg) or a mAb (Santa Cruz, 1.5 μg) at 4°C for 12 h. Protein A–Sepharose was then added. The immunoprecipitates were washed three times with the lysis buffer without SDS or Na deoxycholate, and incubated with an assay buffer containing 50 mM HEPES (pH 7.5), 0.1 mM EDTA, 10 mM $MnCl_2$, 0.015% Brij 35, 25 μM cold ATP, 5 mM $MgCl_2$ and 1 μCi of [γ-^{32}P]ATP at 37°C for 20 min. In some experiments, Raytide (10 μg) was included in the reaction. For the autophosphorylation assay, the reaction was terminated by the addition of SDS loading buffer on ice. The samples were analyzed using a 10% gel. In some experiments, the gel was treated with 1 M KOH to eliminate background serine/threonine phosphorylation. The gel was then dried and subjected to autoradiography. Results were analyzed by densitometry. For the tyrosine kinase assay toward Raytide, the reaction was terminated by spotting the sample onto phosphocellulose units (Pierce). The unit was spun twice with 75 mM phosphoric acid and the radioactivity remaining on the unit was counted.

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References

Abdellatif,M., MacLellan,W.R. and Schneider,M.D. (1994) p21 Ras as a governor of global gene expression. *J. Biol. Chem.*, **269**, 15423–15426.

al-Alawi,N., Rose,D.W., Buckmaster,C., Ahn,N., Rapp,U., Meinkoth,J. and Feramisco,J.R. (1995) Thyrotropin-induced mitogenesis is Ras dependent but appears to bypass the Raf-dependent cytoplasmic kinase cascade. *Mol. Cell. Biol.*, **15**, 1162–1168.

Alblas,J., van Corven,E.J., Hordijk,P.L., Milligan,G. and Moolenaar,W.H. (1993) G_i-mediated activation of the $p21^{ras}$-mitogen-activated protein kinase pathway by α_2-adrenergic receptors expressed in fibroblasts. *J. Biol. Chem.*, **268**, 22235–22238.

Allen,J.S., Gaa,S.T. and Rogers,T.B. (1988) Changes in expression of a functional G_i protein in cultured rat heart cells. *Am. J. Physiol.*, **255**, C51–C59.

Avruch,J., Zhang,X. and Kyriakis,J. (1994) Raf meets Ras: completing the framework of a signal transduction pathway. *Trends Biochem. Sci.*, **19**, 279–283.

Basu,T., Warne,P.H. and Downward,J. (1994) Role of Shc in the

activation of Ras in response to epidermal growth factor and nerve growth factor. *Oncogene*, **9**, 3483–3491.
Blenis,J. (1993) Signal transduction via the MAP kinases: proceed at your own RSK. *Proc. Natl Acad. Sci. USA*, **90**, 5889–5892.
Boguski,M.S. and McCormick,F. (1993) Proteins regulating Ras and its relatives. *Nature*, **366**, 643–654.
Buday,L. and Downward,J. (1993a) Epidermal growth factor regulates $p21^{ras}$ through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. *Cell*, **73**, 611–620.
Buday,L. and Downward,J. (1993b) Epidermal growth factor regulates the exchange rate of guanine nucleotides on $p21^{ras}$ in fibroblasts. *Mol. Cell. Biol.*, **13**, 1903–1910.
Burgering,B.M. and Bos,J.L. (1995) Regulation of Ras-mediated signalling: more than one way to skin a cat. *Trends Biochem. Sci.*, **20**, 18–22.
Cazaubon,S.M., Ramos-Morales,F., Fischer,S., Schweighoffer,F., Strosberg,A.D. and Couraud,P.-O. (1994) Endothelin induces tyrosine phosphorylation and GRB2 association of shc in astrocytes. *J. Biol. Chem.*, **269**, 24805–24809.
Cook,S.J., Rubinfeld,B., Albert,I. and McCormick,F. (1993) RapV12 antagonizes Ras-dependent activation of ERK1 and ERK2 by LPA and EGF in Rat-1 fibroblasts. *EMBO J.*, **12**, 3475–3485.
Cooper,J.A. and Howell,B. (1993) The when and how of Src regulation. *Cell*, **73**, 1051–1054.
Crespo,P., Xu,N., Simonds,W.F. and Gutkind,J.S. (1994) Ras-dependent activation of MAP kinase pathway mediated by G-protein βγ subunits. *Nature*, **369**, 418–420.
Downward,J., Graves,J.D., Warne,P.H., Rayter,S. and Cantrell,D.A. (1990) Stimulation of $p21^{ras}$ upon T-cell activation. *Nature*, **346**, 719–723.
Egan,S.E., Giddings,B.W., Brooks,M.W., Buday,L., Sizeland,A.M. and Weinberg,R.A. (1993) Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. *Nature*, **363**, 45–51.
Furth,M.E., Davis,L.J., Fleurdelys,B. and Scolnick,E.M. (1982) Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus and of the cellular ras gene family. *J. Virol.*, **43**, 294–304.
Gutowski,S., Smrcka,A., Nowak,L., Wu,D.G., Simon,M. and Sternweis,P.C. (1991) Antibodies to the alpha q subfamily of guanine nucleotide-binding regulatory protein alpha subunits attenuate activation of phosphatidylinositol 4,5-bisphosphate hydrolysis by hormones. *J. Biol. Chem.*, **266**, 20519–20524.
Heldin,C.H. (1995) Dimerization of cell surface receptors in signal transduction. *Cell*, 80, 213–223.
Herskowitz,I. (1995) MAP kinase pathways in yeast: for mating and more. *Cell*, **80**, 187–97.
Hordijk,P.L., Verlaan,I., van Corven,E.J. and Moolenaar,W.H. (1994) Protein tyrosine phosphorylation induced by lysophosphatidic acid in Rat-1 fibroblasts. Evidence that phosphorylation of map kinase is mediated by the G_i-$p21^{ras}$ pathway. *J. Biol. Chem.*, **269**, 645–651.
Hunter,T. (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell*, **80**, 225–236.
Kai,H., Griendling,K.K., Lassegue,B., Ollerenshaw,J.D., Runge,M.S. and Alexander,R.W. (1994) Agonist-induced phosphorylation of the vascular type 1 angiotensin II receptor. *Hypertension*, **24**, 523–527.
Koch,W.J., Hawes,B.E., Allen,L.F. and Lefkowitz,R.J. (1994) Direct evidence that G_i-coupled receptor stimulation of mitogen-activated protein kinase is mediated by $G_{\beta\gamma}$ activation of $p21^{ras}$. *Proc. Natl Acad. Sci. USA*, **91**, 12706–12710.
Kypta,R.M., Goldberg,Y., Ulug,E.T. and Courtneidge,S.A. (1990) Association between the PDGF receptor and members of the *src* family tyrosine kinase. *Cell*, **62**, 481–492.
LaMorte,V.J., Kennedy,E.D., Collins,L.R., Goldstein,D., Harootunian, A.T., Brown,J.H. and Feramisco,J.R. (1993) A requirement for ras protein function in thrombin-stimulated mitogenesis in astrocytoma cells. *J. Biol. Chem.*, **268**, 19411–19415.
Langhans-Rajasekaran,S.A., Wan,Y. and Huang,X.-Y. (1995) Activation of Tsk and Btk tyrosine kinases by G protein βγ subunits. *Proc. Natl Acad. Sci. USA*, **92**, 8601–8605.
Lev,S., Moreno,H., Martinez,R., Canoll,P., Peles,E., Musacchio,J.M., Plowman,G.D., Rudy,B. and Schlessinger,J. (1995) Protein tyrosine kinase PYK2 involved Ca^{2+}-induced regulation of ion channel and MAP kinase functions. *Nature*, **376**, 737–744.
Marrero,M.B., Schieffer,B., Paxton,W.G., Heerdt,L., Berk,B.C., Delafontaine,P. and Bernstein,K.E. (1995) Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. *Nature*, **375**, 247–250.
McCormick,F. (1994) Activators and effectors of ras p21 proteins. *Curr. Opin. Genet. Dev.*, **4**, 71–76.
McGlade,J., Cheng,A., Pelicci,G., Pelicci,P.G. and Pawson,T. (1992) Shc proteins are phosphorylated and regulated by the v-Src and v-Fps protein-tyrosine kinases. *Proc. Natl Acad. Sci. USA*, **89**, 8869–8873.
Medema,R.H., de Vries-Smits,A.M., van der Zon,G.C., Maassen,J.A. and Bos,J.L. (1993) Ras activation by insulin and epidermal growth factor through enhanced exchange of guanine nucleotides on $p21^{ras}$. *Mol. Cell. Biol.*, **13**, 155–162.
Neer,E.J. (1995) Heterotrimeric G proteins: organizers of transmembrane signals. *Cell*, **80**, 249–257.
Ohmichi,M., Sawada,T., Kanda,Y., Koike,K., Hirota,K., Miyake,A. and Saltiel,A.R. (1994) Thyrotropin-releasing hormone stimulates MAP kinase activity in GH3 cells by divergent pathways. *J. Biol. Chem.*, **269**, 3783–3788.
Paxton,W.G., Marrero,M.B., Klein,J.D., Delafontaine,P., Berk,B.C. and Bernstein,K.E. (1994) The angiotensin II AT_1 receptor is tyrosine and serine phosphorylated and can serve as a substrate for the *src* family of tyrosine kinases. *Biochem. Biophys. Res. Commun.*, **200**, 260–267.
Pelicci,G. *et al.* (1992) A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell*, **70**, 93–104.
Ravichandran,K.S., Lorenz,U., Shoelson,S.E. and Burakoff,S.J. (1995) Interaction of Shc with Grb2 regulates association of Grb2 with mSOS. *Mol. Cell. Biol.*, **15**, 593–600.
Rickles,R.J., Botfield,M.C., Weng,Z., Taylor,J.A., Green,O.M., Brugge,J.S. and Zoller,M.J. (1994) Identification of Src, Fyn, Lyn, PI3K and Abl SH3 domain ligands using phage display libraries. *EMBO J.*, **13**, 5598–5604.
Rodriguez-Viciana,P., Warne,P.H., Dhand,R., Vanhaesebroeck,B., Gout,I., Fry,M.J., Waterfield,M.D. and Downward,J. (1994) Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature*, **370**, 527–532.
Rozakis-Adcock,M. *et al.* (1992) Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature*, **360**, 689–692.
Sadoshima,J. and Izumo,S. (1993a) Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism. *EMBO J.*, **12**, 1681–1692.
Sadoshima,J. and Izumo,S. (1993b) Molecular characterization of angiotensin II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts: a critical role of the AT_1 receptor subtype. *Circ. Res.*, **73**, 413–423.
Sadoshima,J. and Izumo,S. (1993c) Signal transduction pathways of angiotensin II induced c-*fos* gene expression in cardiac myocytes *in vitro*: roles of phospholipid-derived second messengers. *Circ. Res.*, **73**, 424–438.
Sadoshima,J., Jahn,L., Takahashi,T., Kulik,T.J. and Izumo,S. (1992) Molecular characterization of the stretch-induced adaptation of cultured cardiac cells: an *in vitro* model of load-induced cardiac hypertrophy. *J. Biol. Chem.*, **267**, 10551–10560.
Sadoshima,J., Xu,Y., Slayter,H.S. and Izumo,S. (1993) Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes *in vitro*. *Cell*, **95**, 977–984.
Sadoshima,J., Qiu,Z., Morgan,J. and Izumo,S. (1995) Angiotensin II and other hypertrophic stimuli mediated by G protein coupled receptors activate tyrosine kinase, MAP kinase and 90 kDa S6 kinase in cardiac myocytes: the critical role of Ca^{2+}-dependent signaling. *Circ. Res.*, **76**, 1–15.
Satoh,T., Nakafuku,M. and Kaziro,Y. (1992) Function of ras as a molecular switch in signal transduction. *J. Biol. Chem.*, **267**, 24149–24152.
Schorb,W., Peeler,T.C., Madigan,N.N., Conrad,K.M. and Baker,K.M. (1994) Angiotensin II-induced protein tyrosine phosphorylation in neonatal rat cardiac fibroblasts. *J. Biol. Chem.*, **269**, 19626–19632.
Shou,C., Wurmser,A., Ling,K., Barbacid,M. and Feig,L.A. (1995) Differential response of the Ras exchange factor, Ras-GRF to tyrosine kinase and G protein mediated signals. *Oncogene*, **10**, 1887–1893.
Songyang,Z. *et al.* (1994) Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRB-2, HCP, SHC, Syk, and Vav. *Mol. Cell. Biol.*, **14**, 2777–2785.
Stein,P.L., Lee,H.M., Rich,S. and Soriano,P. (1992) pp59fyn mutant mice display differential signaling in thymocytes and peripheral T cells. *Cell*, **70**, 741–750.

Taniguchi,T. (1995) Cytokine signaling through nonreceptor protein tyrosine kinases. *Science*, **268**, 251–255.

Thorburn,A., Thorburn,J., Chen,S.-Y., Powers,S., Shubeita,H.E., Feramisco,J.R. and Chien,K.R. (1993) HRas-dependent pathways can activate morphological and genetic markers of cardiac muscle cell hypertrophy. *J. Biol. Chem.*, **268**, 2244–2249.

Tsai,M.H., Yu,C.L., Wei,F.S. and Stacey,D.W. (1989) The effect of GTPase activating protein upon ras is inhibited by mitogenically responsive lipids. *Science*, **243**, 522–526.

van Biesen,T., Hawes,B.E., Luttrell,D.K., Krueger,K.M., Touhara,K., Porfiri,E., Sakaue,M., Luttrell,L.M. and Lefkowitz,R.J. (1995) Receptor-tyrosine-kinase- and $G_{\beta\gamma}$-mediated MAP kinase activation by a common signaling pathway. *Nature*, **376**, 781–784.

van Corven,E.J., Horduk,P.L., Medema,R.H., Bos,J.L. and Moolenaar,W.H. (1993) Pertussis toxin-sensitive activation of $p21^{ras}$ by G protein-coupled receptor agonists in fibroblasts. *Proc. Natl Acad. Sci. USA*, **90**, 1257–1261.

van der Geer,P., Wiley,S., Lai,V.K.-M., Olivier,J.P., Gish,G.D., Stephens,R., Kaplan,D., Shoelson,S. and Pawson,T. (1995) A conserved amino-terminal Shc domain binds to phosphotyrosine motifs in activated receptors and phosphopeptides. *Curr. Biol.*, **5**, 404–412.

Vojtek,A.B., Hollenberg,S.M. and Cooper,J.A. (1993) Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell*, **74**, 205–214.

Weiss,A. and Littman,D.R. (1994) Signal transduction by lymphocyte antigen receptors. *Cell*, **76**, 263–274.

Weng,Z., Thomas,S.M., Rickles,R.J., Taylor,J.A., Brauer,A.W., Seidel-Dugan,C., Michael,W.M., Dreyfuss,G. and Brugge,J.S. (1994) Identification of src, fyn and lyn SH3-binding proteins: implications for a function of SH3 domains. *Mol. Cell. Biol.*, **14**, 4509–4521.

Winitz,S., Russell,M., Qian,N.-X., Gardner,A., Dwyer,L. and Johnson,G.L. (1993) Involvement of ras and raf in the G_i-coupled acetylcholine muscarinic m2 receptor activation of mitogen-activated protein (MAP) kinase kinase and MAP kinase. *J. Biol. Chem.*, **268**, 19196–19199.

Yu,H., Rosen,M.K., Shin,T.B., Seidel-Dugan,C., Brugge,J.S. and Schreiber,S.L. (1992) Solution structure of the SH3 domain of Src and identification of its ligand-binding site. *Science*, **258**, 1665–1668.

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