Angiotensin II stimulates serine phosphorylation of the adaptor protein Nck: physical association with the serine/threonine kinases Pak1 and casein kinase I

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Nck is a small adaptor protein consisting exclusively of three SH3 domains and one SH2 domain. Nck is thought to have an important role in cell signalling by coupling receptor tyrosine kinases, via its SH2 domain, to downstream SH3-binding effectors. We report here that angiotensin II, working through the AT₁ receptor subtype, stimulates the phosphorylation of Nck in rat aortic smooth muscle cells. Phosphopeptide mapping analysis revealed that Nck is phosphorylated on four peptides containing exclusively phosphoserine in quiescent cells. Treatment with angiotensin II resulted in increased phosphorylation of these four peptides, without the appearance of new phosphopeptides. We show that Nck, via its SH3 domains, specifically binds three major phosphoproteins of 95, 82 and 66 kDa both *in vitro* and in intact cells. Notably, the phosphorylation of these Nck-binding

proteins was found to increase in parallel with that of Nck on stimulation by angiotensin II. One candidate for the 66 kDa phosphoprotein is the serine/threonine kinase p21-activated kinase 1 (Pak1), which was found to form a stable complex with Nck in aortic smooth muscle cells. We have also identified the γ 2 isoform of casein kinase I as another protein kinase that associates with Nck in these cells. These findings indicate that Nck is a target of G-protein-coupled receptors and suggest a role for Pak1 and casein kinase I- γ 2 in downstream signalling or regulation of the AT₁ receptor.

Key words: angiotensin receptor, protein kinase, signal transduction, smooth muscle.

INTRODUCTION

The transduction of extracellular signals from the plasma membrane to intracellular targets is co-ordinated through specific protein–protein interactions. These interactions are mediated by conserved protein modules that serve to recruit effector enzymes into signalling networks or to localize enzymes close to their substrates (reviewed in [1,2]). Several modular binding domains have been identified in signalling molecules of diverse functions such as protein kinases, lipid kinases, phospholipases or transcription factors. These modules are also found in a special class of signalling proteins, termed adaptors, that are devoid of catalytic activity and act by providing additional docking sites for signalling molecules [1,2]. Like Grb2, Shc and Crk, Nck [3] belongs to this family of SH2/SH3-domain-containing adaptors.

Nck is a 47 kDa protein consisting of three SH3 domains and one SH2 domain, which is ubiquitously expressed in tissues and cell lines [3,4]. Consistently with its role as an adaptor, Nck has been shown to interact via its SH2 domain with activated receptor tyrosine kinases (e.g. receptors for epidermal growth factor [4], platelet-derived growth factor [4] and vascular endothelial growth factor [5]) or with the docking proteins insulin receptor substrate 1 [6] and p62^{dok} [7]. In recent years, several signalling molecules have been reported to interact with the SH3 domains of Nck. These include Cbl [8], SOS [9], the Wiskott–Aldrich syndrome protein WASP [10], the RNA-binding protein SAM68 [11] and several serine/threonine kinases such as the Cdc42/Rac-activated Paks [12–14], Nck-associated kinase [15], protein kinase C-related kinase 2 [16], Nck-interacting kinase

[17] and casein kinase I- γ 2 [18]. Thus Nck is believed to participate in signal transduction downstream of activated membrane receptors by mediating the assembly of multiprotein signalling complexes. However, relatively little is known about the regulation and specific biological functions of Nck. The adaptor protein becomes phosphorylated on serine/threonine and, in some instances, on tyrosine residues in response to the activation of receptor tyrosine kinases, T-cell and immunoglobulin receptors, phorbol esters and cAMP [4,19,20]. The overexpression of Nck leads to transformation of rodent fibroblasts and tumour formation in nude mice [4,21] and inhibits nerve-growth-factor-induced neurite outgrowth in PC-12 cells [22]. Genetic studies in *Drosophila* revealed that the Nck homologue Dock has a crucial role in photoreceptor cell axon guidance and targeting [23]. In *Xenopus laevis*, the expression of mutated Nck was shown to affect the migration of the mesoderm cells and to respecify mesodermal cell fate [24]. These findings, together with the observation that Nck binds to downstream effectors of Rho-family GTPases, point to a role of Nck in coupling membrane receptors to reorganization of the cytoskeleton.

Angiotensin II (Ang II), the primary effector of the renin–angiotensin system, is an important regulator of vascular function. In addition to its potent vasoconstrictor action, Ang II causes hypertrophy of vascular smooth muscle cells (SMC) [25–27] and induces vascular cell migration [28]. These actions of the hormone are mediated by the AT₁ receptor subtype, a member of the G-protein-coupled receptor superfamily. Binding of Ang II to the AT₁ receptor stimulates the activity of phospholipase C (PLC) to produce the second messengers Ins(1,4,5)P₃

Abbreviations used: Ang II, angiotensin II; CKI, casein kinase I; CKI-7, *N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulphonamide; GST, glutathione S-transferase; Pak, p21-activated kinase; PLC, phospholipase C; SMC, smooth muscle cells.

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and diacylglycerol, and inhibits the activity of adenylate cyclase [29]. Receptor occupancy also leads to increased tyrosine phosphorylation of multiple cellular proteins [30]. These early signals subsequently lead to the activation of multiple serine/threonine kinases, which include protein kinase C [31], p70 S6 kinase [27] and the mitogen-activated protein kinases ERK1 and ERK2 [32,33].

Here we report that activation of the G-protein-coupled AT₁ receptor by Ang II stimulates the phosphorylation of Nck on multiple serine residues in rat aortic SMC. Moreover, we show that Nck, via its SH3 domains, is bound to multiple cellular phosphoproteins whose phosphorylation also increases in response to Ang II. We have identified two of the Nck-associated proteins as the serine/threonine kinases Pak1 and casein kinase I (CKI)- γ 2.

EXPERIMENTAL

Materials and antibodies

The source of materials has been described previously [27]. *N*-(2-aminoethyl)-5-chloroisoquinoline-8-sulphonamide (CKI-7) was obtained from Seikagaku Corp. GTP was from Sigma. Recombinant glutathione S-transferase (GST) fusion proteins of various domains of human Nck (GST-SH2, residues 282–377; GST-SH3, residues 1–251; GST-Nck, full-length cDNA) and of the SH3 domain of PLC- γ (GST-PLC- γ SH3, residues 798–850) were expressed in *Escherichia coli* and immobilized on glutathione-agarose beads in accordance with the manufacturer's instructions (Pharmacia). Rabbit polyclonal antibodies against Nck and CKI- γ 2 have been described [18]. The anti-Pak1 antibody (sc-882) was obtained from Santa Cruz Biotechnology.

Cell culture

Rat aortic SMC were cultured and synchronized in the quiescent state as previously described [27].

Labelling with ³²P and immunoprecipitation of Nck

Quiescent rat aortic SMC in 10 cm diameter Petri dishes were metabolically labelled for 5 h at 37 °C in bicarbonate- and phosphate-free Hepes-buffered Dulbecco's modified Eagle's medium containing 0.2–0.5 mCi/ml [³²P]phosphoric acid. The cells were stimulated by the addition of 100 nM Ang II to the medium for the indicated durations and quickly washed twice with ice-cold PBS. Cell lysates were prepared in Triton X-100 lysis buffer [50 mM Tris/HCl (pH 7.4)/100 mM NaCl/50 mM NaF/5 mM EDTA/40 mM β -glycerophosphate/1 mM sodium orthovanadate/100 μ M PMSF/1 μ M leupeptin/1 μ M pepstatin A/1 % (v/v) Triton X-100] and clarified by centrifugation at 13 000 *g* for 5 min at 4 °C [27]. For immunoprecipitation of Nck, the lysates were precleared for 1 h with 5 μ l of normal rabbit serum and incubated for 4 h at 4 °C with 10 μ l of anti-Nck antibody preadsorbed on Protein A-Sepharose beads. Immune complexes were washed five times with Triton X-100 lysis buffer. For binding experiments *in vitro* with GST fusion proteins, cell lysates were incubated for 1 h at 4 °C with 3 μ g of the individual GST fusion proteins. Bound proteins were washed three times with HNTG buffer [20 mM Hepes (pH 7.5)/150 mM NaCl/0.1 % (v/v) Triton X-100/10 % (v/v) glycerol/10 μ g/ml leupeptin/0.1 mM PMSF/200 μ M sodium orthovanadate]. All samples were heated in Laemmli denaturing buffer and analysed by SDS/PAGE [7.5 % (w/v) gel].

Peptide mapping and phosphoamino acid analysis

³²P-labelled Nck immunoprecipitates from metabolically labelled cells were resolved by SDS/PAGE [7.5 % (w/v) gel]. The proteins were then transferred electrophoretically to PVDF membranes (Millipore) in 25 mM Tris/192 mM glycine/20 % (v/v) methanol and detected by autoradiography. The labelled band corresponding to Nck was excised from the membrane and subjected to phosphoamino acid analysis or to phosphopeptide mapping as described previously [34].

Immunoblot analysis

Quiescent aortic SMC were stimulated with Ang II for the indicated durations at 37 °C. Cell lysates were prepared and equal amounts of proteins were subjected to immunoprecipitation with Nck-specific antibody as described above. The eluted proteins were resolved by SDS/PAGE and transferred to Hybond-C nitrocellulose membrane (Amersham) in 25 mM Tris/192 mM glycine. For immunoblotting of Nck and Pak1, the membrane was blocked for 1 h at 37 °C in TBS/0.1 % (v/v) Tween 20/5 % (w/v) non-fat dried milk, then incubated for 1 h at 25 °C with Nck- or Pak1-specific antibody (diluted 1:500) in blocking buffer. The membrane was then washed six times with TBS/0.1 % (v/v) Tween 20 before incubation for 1 h at 25 °C with horseradish peroxidase-conjugated goat anti-rabbit IgG diluted 1:10 000 in blocking buffer. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham). For immunoblotting of CKI- γ 2, the membrane was incubated for 90 min at 25 °C with CKI- γ 2-specific antibody diluted 1:250 in blocking buffer.

Casein kinase assay *in vitro*

Quiescent aortic SMC were stimulated with Ang II for the indicated durations and Nck was immunoprecipitated as described above. The immune complexes were washed three times with Triton X-100 lysis buffer, once with kinase assay buffer [20 mM Hepes (pH 7.5)/1 mM dithiothreitol/5 mM MgCl₂/10 mM β -glycerophosphate], then resuspended in 30 μ l of kinase assay buffer containing 10 μ g of α -casein as substrate. The CKI selective inhibitor CKI-7 (100 μ M) [35] or GTP (100 μ M) were added or not to the assay. The reaction was initiated by the addition of ATP (20 μ M, 10 μ Ci [γ -³²P]ATP), incubated for 20 min at 30 °C, and stopped by the addition of 6 $\times$ Laemmli sample buffer. The samples were boiled and analysed by SDS/PAGE and autoradiography.

RESULTS

Ang II rapidly increases phosphorylation of Nck in aortic SMC

To explore the role of the adaptor protein Nck in G-protein-coupled receptor signalling, we first examined the ability of Ang II to regulate the phosphorylation of Nck in rat aortic SMC. The cells were metabolically labelled with [³²P]_i and treated with Ang II for different durations; then Nck was immunoprecipitated from cell lysates by using a specific antibody. Figure 1 shows that Nck is constitutively phosphorylated in quiescent cells and that Ang II increases the phosphorylation of the protein. Interestingly, detailed kinetic analysis revealed that Ang II-dependent phosphorylation of Nck was biphasic. There was a rapid increase in Nck phosphorylation, which reached a maximum at 1 min and rapidly returned to the control level by 10 min. This was followed by a second induction of phosphorylation at 30 min, which persisted for up to at least 120 min. The same amount of Nck protein was immunoprecipitated in all the samples. The maximal

Figure 1 Ang II stimulates the phosphorylation of Nck in aortic SMC through activation of the AT₁ receptor

Quiescent rat aortic SMC were incubated with 0.2 mCi/ml [³²P]phosphoric acid for 5 h before treatment with 100 nM Ang II for the indicated durations. Cell lysates were prepared and subjected to immunoprecipitation with Nck-specific antiserum preadsorbed on Protein A-Sepharose beads. The immunoprecipitated proteins were resolved by SDS/PAGE [7.5% (w/v) gel]. After being fixed and dried, the gel was exposed for autoradiography. **(A)** Time course of Ang II stimulation of Nck phosphorylation. An autoradiogram of the gel is shown (upper panel). The numbers in parentheses show the relative radioactivity incorporated into the Nck protein (control sample, 302 c.p.m.). The content of Nck protein in the immunoprecipitated samples was analysed by immunoblotting with Nck-specific antibody (αNck; lower panel). **(B)** Effect of the AT₁ selective receptor antagonist losartan. In this experiment, the cells were pretreated for 30 min with medium alone or with 10 μM losartan before stimulation or not with Ang II for 1 min. The positions of Nck and of IgG heavy chain are indicated by an arrow. The bracket indicates the position of the phosphoproteins that immunoprecipitated with Nck. The results are representative of three independent experiments. The positions of molecular mass markers are indicated (in kDa) at the left.

level of Nck phosphorylation observed after treatment with Ang II was 2–2.5-fold over basal. Incubation of the cells with the AT₁-selective antagonist losartan completely suppressed the Ang II-induced phosphorylation of Nck (Figure 1B), demonstrating that the effect of Ang II is mediated by the AT₁ subtype of receptor. These results suggest that Nck might have a role in signalling pathways downstream of the G-protein-coupled AT₁ receptor.

Ang II stimulates the phosphorylation of Nck on multiple serine residues

The phosphorylation sites in Nck were further investigated by phosphoamino acid analysis and tryptic phosphopeptide mapping. Analysis of the total protein revealed that Nck was phosphorylated exclusively on serine residues in both quiescent and Ang II-treated cells (Figure 2B). These data are consistent with the results of anti-phosphotyrosine immunoblotting studies, which failed to detect phosphotyrosine immunoreactivity in the Nck protein (results not shown). The phosphopeptide map of Nck isolated from growth-arrested cells consisted of four major spots (Figure 2C). This pattern of phosphopeptides observed in the resting condition is similar to that observed in A431 cells [4,20] and NIH 3T3 cells [19]. The increased phosphorylation of Nck induced by Ang II was associated with an augmentation of the phosphate content in each of these peptides, without the appearance of new phosphopeptides (Figure 2D). Analysis of each individual spot (numbered 1–4) showed the exclusive presence of phosphoserine (results not shown).

SH3 domains of Nck interact with multiple cellular phosphoproteins: identification of Pak1

Nck has been reported to interact with multiple signalling proteins via its SH2 and SH3 domains. Results in Figure 1(A) clearly indicate that Nck immunoprecipitates with several phosphorylated proteins in aortic SMC. The three most prominent phosphoproteins migrated with apparent molecular masses of 92–98 (pp95), 80–84 (pp82) and 64–68 (pp66) kDa. Notably, the phosphorylation of Nck-associated proteins, in response to stimulation with Ang II, followed a similar kinetics to Nck phosphorylation. This might result either from an increase in their association with Nck or from a modification of their phosphorylation state after treatment with Ang II. To determine which domain of Nck was responsible for association to these phosphoproteins, we performed binding experiments *in vitro* with GST fusion proteins containing distinct domains of Nck. Lysates from ³²P-labelled aortic SMC stimulated with Ang II were incubated with the fusion proteins immobilized to glutathione-agarose beads; the bound protein complexes were analysed by SDS/PAGE and autoradiography (Figure 3). No binding of labelled proteins to Nck SH2 domain or to control GST was detected in this assay. In contrast, full-length Nck or the three SH3 domains of Nck bound to the same three phosphoproteins detected in co-immunoprecipitation experiments and to additional phosphoproteins of 110–115 and 170 kDa. Intriguingly, we always observed greater binding to GST–Nck SH3 and it is possible that the SH2 domain negatively regulates binding, through steric hindrance or another mechanism. Thus these results indicate that the three major phosphoproteins interact specifically with the SH3 domains of Nck both *in vitro* and in intact cells.

It has been reported recently [36] that Ang II stimulates the phosphorylation of proteins with molecular masses of 97, 84, 60 and 47 kDa that are co-precipitated by antibodies against PLC-

Figure 2 Ang II stimulates the phosphorylation of Nck on multiple serine residues

Quiescent aortic SMC were labelled with 0.5 mCi/ml [32 P]phosphoric acid for 5 h and then stimulated or not (cont) with 100 nM Ang II for 1 min. Nck immunoprecipitates were resolved by SDS/PAGE and the labelled proteins were transferred electrophoretically to PVDF membrane. (A) An autoradiogram of the membrane. (B) The 32 P-labelled Nck bands were excised from the PVDF membrane and subjected to partial acid hydrolysis. The phosphorylated amino acids were separated by one-dimensional thin-layer electrophoresis. pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine. (C, D) The 32 P-labelled Nck bands were digested extensively with trypsin and the resulting phosphopeptides were separated by thin-layer electrophoresis followed by ascending chromatography. The phosphopeptides were labelled from 1 to 4. The arrow denotes the position of sample application. The amounts of radioactivity applied to each plate were 253 c.p.m. (C) and 514 c.p.m. (D). Similar results were obtained in three separate experiments.

Figure 3 SH3 domains of Nck bind to multiple phosphoproteins *in vitro*

Quiescent aortic SMC were labelled with 0.2 mCi/ml [32 P]phosphoric acid for 5 h before stimulation with 100 nM Ang II for 1 min. Cell lysates were prepared and incubated with purified GST fusion proteins containing different domains of Nck. After being washed, bound proteins were resolved by SDS/PAGE [7.5% (w/v) gel] and detected by autoradiography. The results are representative of three independent experiments. The positions of molecular mass markers are indicated (in kDa) at the left.

γ 1. The 47 kDa protein was identified as Nck by immunoblotting analysis. Earlier studies on PLC- γ 1 regulation also showed that PLC- γ 1 immunoprecipitates from various cells labelled with

[32 P] P_i contain phosphoproteins of approx. 100, 84 and 47 kDa [19,20,37]. Subsequent investigation [19,20] demonstrated that certain monoclonal antibodies raised against PLC- γ 1 cross-react with Nck, most probably because the two proteins share a high amino acid identity in their respective SH3 domains. Therefore it was important to verify whether the three phosphoproteins present in Nck immunoprecipitates are bound to Nck rather than to PLC- γ 1. We first confirmed that our Nck antibody did not cross-react with PLC- γ 1 and further showed that PLC- γ 1 is not detected in Nck immunoprecipitates (results not shown). Then we determined whether the SH3 domain of PLC- γ 1 can interact directly with the same phosphoproteins *in vitro*. Purified GST fusion proteins containing the SH3 domains of Nck or the SH3 domain of PLC- γ 1 were immobilized on glutathione-agarose beads and incubated with lysates from 32 P-labelled aortic SMC stimulated with Ang II. Bound proteins were analysed by SDS/PAGE and autoradiography; in contrast with Nck, no binding of PLC- γ 1-SH3 to cellular phosphoproteins was detected in these cells (Figure 4). We conclude from these findings that the pp95, pp82 and pp66 phosphoproteins found in Nck immunoprecipitates are not complexed with PLC- γ 1.

One possible candidate for the 66 kDa phosphoprotein is the Ste20-related serine/threonine kinase Pak1, which was recently shown to bind Nck in some cell types [13,14]. The interaction between Nck and Pak1 is mediated by the second SH3 domain of Nck and the N-terminus of Pak1 [13,14]. To determine whether Nck is complexed with Pak1 in vascular SMC, cellular lysates were subjected to immunoprecipitation with anti-Nck antibody and analysed by immunoblotting with Pak1-specific antibody. Pak1 co-precipitated with Nck in both quiescent and Ang II-stimulated cells, indicating that the two proteins are constitutively associated in aortic SMC (Figure 5). Notably, the amount of Pak1 complexed with Nck doubled on stimulation with Ang II. In agreement with the idea that Pak1 might

Figure 4 Lack of binding of PLC- γ 1 SH3 domain to cellular phosphoproteins *in vitro*

Quiescent aortic SMC were labelled with 0.2 mCi/ml [³²P]phosphoric acid for 5 h before stimulation with 100 nM Ang II for 1 min. Cell lysates were prepared and incubated with purified GST fusion proteins containing the three SH3 domains of Nck or the SH3 domain of PLC- γ 1. After being washed, bound proteins were resolved by SDS/PAGE [7.5% (w/v) gel] and detected by autoradiography. The results are representative of two independent experiments. The positions of molecular mass markers are indicated (in kDa) at the left.

Figure 5 Association of Nck with Pak1 in aortic SMC

Quiescent aortic SMC were stimulated or not with Ang II for the indicated durations. Cell lysates were prepared and subjected to immunoprecipitation with Nck-specific antibody preadsorbed on Protein A-Sepharose beads. The immunoprecipitates were analysed by immunoblotting with Pak1-specific antibody (α PAK). An aliquot of total cell lysate was run alongside the precipitates as control. The position of Pak1, which migrated as a doublet, is indicated by an arrow.

correspond to pp66, we found that Ang II also increased the phosphorylation of Pak1 in aortic SMC (results not shown).

Nck associates with CKI- γ 2 in aortic SMC

The isoform γ 2 of CKI was recently shown to be constitutively associated with Nck in transformed rat hepatocytes over-expressing the insulin receptor [18]. To determine whether CKI- γ 2 is associated with Nck in aortic SMC, cellular lysates were subjected to immunoprecipitation with Nck antibody and immunoblotted with CKI- γ 2-specific antibody. As shown in Figure 6(A), CKI- γ 2 was detected in Nck immunoprecipitates from

Figure 6 Nck associates with CKI- γ 2 in aortic SMC

(A) Quiescent aortic SMC were stimulated or not with 100 nM Ang II for the indicated durations. Cell lysates were prepared and subjected to immunoprecipitation with Nck-specific antiserum (α Nck) preadsorbed on Protein A-Sepharose beads. The immunoprecipitated proteins and an aliquot of total cell lysate were analysed by immunoblotting with a CKI- γ 2-specific antibody. The position of CKI- γ 2 (70–75 kDa) is indicated by an arrow. (B) Immune complex kinase assay of Nck-associated CKI activity. Quiescent aortic SMC were stimulated or not with Ang II for the indicated durations and cell lysates were subjected to immunoprecipitation with Nck antiserum. Immune complexes were washed and CKI phosphotransferase activity was assayed directly with α -casein as substrate. (C) Quiescent aortic SMC were stimulated or not with Ang II for 5 min and CKI activity was assayed as in (B). The specificity of the kinase assay was determined by the addition of GTP or the selective inhibitor CKI-7 to the reaction. The reaction products were separated on 12% polyacrylamide gel and detected by autoradiography. Numbers in parentheses are the relative CKI activity as quantified by densitometric analysis of the α -casein band. Similar results were obtained in three separate experiments.

both quiescent and Ang II-stimulated cells. No significant change in the amount of CKI- γ 2 was observed after treatment with Ang II. Immune complexes of normal rabbit serum did not contain the CKI- γ 2 protein (results not shown). We next investigated whether Nck-associated CKI- γ 2 displays kinase activity *in vitro*. The phosphotransferase activity of CKI- γ 2 was measured directly on Nck immunoprecipitates with α -casein as substrate in the presence or absence of CKI-7, a selective inhibitor of CKI [35], or GTP, which can be used as a phosphate donor by CKII but not CKI. The results of these experiments showed that Nck immunoprecipitates with CKI activity, because CKI-7 completely blocked α -casein phosphorylation, whereas the addition of GTP had little effect (Figures 6B and 6C). Interestingly, we found that Nck-associated CKI activity was weakly increased (1.6-fold) on stimulation with Ang II for 5 min. This increase is likely to have been the result of a net activation of CKI- γ 2, because the amount of CKI- γ 2 protein associated to Nck did not change after treatment with Ang II (Figure 6A).

DISCUSSION

Nck belongs to the adaptor family of signalling proteins. Small adaptor proteins such as Grb2, Crk and Nck are composed almost exclusively of SH2 and SH3 domains and are believed to

have an important role in signal transduction by coupling receptors with intrinsic or associated tyrosine kinase activity, bound by SH2 domains, to downstream SH3-associated effectors. Nck has been shown to be recruited, via its SH2 domain, to activated tyrosine kinase receptors [4,5] and to intermediate docking proteins [6]. However, several potential downstream targets that interact with the SH3 domains of Nck have been identified in recent years (see the Introduction section). The presence of three SH3 domains in Nck and the relative binding selectivity of SH3 domains suggest that Nck might cluster several distinct signalling molecules. The adaptor role of Nck might also be regulated by phosphorylation, as Nck becomes phosphorylated on serine, threonine and tyrosine residues after the activation of receptor tyrosine kinases, T-cell receptor or membrane IgM receptor, and in v-Src-transformed cells [4,19–21]. We report here that Ang II, working through the AT₁ receptor, stimulates the serine phosphorylation of Nck in aortic SMC. These findings support the idea that Nck might act as a universal adaptor protein linking various families of cell-surface receptors to downstream signalling pathways or receptor regulatory mechanisms.

The signalling events by which growth factors induce the phosphorylation of Nck and the identity of the protein kinases involved are not known. We observed that, in quiescent aortic SMC, Nck is phosphorylated on four major peptides that contain exclusively phosphoserine. Treatment with Ang II resulted in increased phosphorylation of these four peptides but did not generate additional phosphopeptides. It is noteworthy that the same four spots are also labelled in tryptic peptide maps of Nck isolated from NIH 3T3 fibroblasts and A431 epidermoid carcinoma cells [4,19,20]; in contrast with the effect of Ang II, however, treatment of NIH 3T3 cells with platelet-derived growth factor or treatment of A431 cells with epidermal growth factor results in the appearance of new phosphopeptides and of phosphotyrosine [19]. The site of tyrosine phosphorylation was located to a single peptide (spot 7 in [19]), which is absent from the map of Nck from Ang II-treated cells. One possible candidate for the serine/threonine kinases that phosphorylate Nck on serine residues is Pak1. It has been reported [14] that Pak1 binds to the second SH3 domain of Nck and phosphorylates Nck on multiple sites *in vitro*. In agreement with this idea, we report here that Nck and Pak1 form a complex in intact aortic SMC. Another candidate for the Nck kinase is CKI- γ 2 (see below).

One important finding of this study is the observation that Nck, via its SH3 domains, associates with three major phosphoproteins whose kinetics of phosphorylation, in response to Ang II stimulation, parallels that of Nck. The interaction of Nck with these phosphoproteins of 95, 82 and 66 kDa was demonstrated both in intact aortic SMC by co-immunoprecipitation studies and in binding experiments *in vitro* with GST fusion proteins. Several groups have reported that immunoprecipitates made with PLC- γ 1 monoclonal antibodies contain three prominent phosphoproteins of approx. 100, 84 and 47 kDa whose phosphorylation increases in response to growth factors [19,20,36,37]. The 47 kDa protein was identified as Nck and it was further shown that Nck and PLC- γ 1 share a common epitope that is recognized by certain PLC- γ 1 monoclonal antibodies [19,20]. This raises the important question of whether the approx. 100 and 84 kDa phosphoproteins found in PLC- γ 1 precipitates, which are similar to the pp95 and pp82 described in this study, are associated with PLC- γ 1 or Nck. The following observations provide compelling evidence that pp95 and pp82 (and also pp66) are specific effectors for Nck. First, PLC- γ 1 is not detected in Nck immunoprecipitates from intact cells. Secondly, a GST fusion protein containing the SH3 domains of Nck

binds to pp95, pp82 and pp66 *in vitro*. Thirdly, the PLC- γ 1 SH3 domain does not detectably interact with these three phosphoproteins.

We have identified the serine/threonine kinase Pak1 as a strong candidate for the 66 kDa phosphoprotein, whereas the identities of pp82 and pp95 remain to be established. Two additional phosphoproteins of 170 and 110–115 kDa bound effectively to GST fusion proteins containing full-length Nck or the three SH3 domains of Nck but were hardly detectable in Nck immunoprecipitates. An attractive candidate for the 170 kDa phosphoprotein is the guanyl nucleotide exchange factor SOS1 [9], whereas the 110–115 kDa protein might correspond to protein kinase C-related kinase 2, a serine/threonine kinase closely related to the Rho effector protein kinase N [16]. The role of Nck binding and phosphorylation in the regulation of these phosphoproteins is intriguing. One possibility is that serine phosphorylation of Nck induces a conformational change in the SH3 domains that increases or stabilizes the binding of Nck to these phosphoproteins. Such a mechanism is suggested by the observation that Pak1 binding to Nck increases on stimulation of the platelet-derived growth factor receptor in Swiss 3T3 cells [14] and activation of the AT₁ receptor in vascular SMC (this study). Alternatively, the phosphorylation of Nck might enhance the specific activity of a bound protein kinase, resulting in the increased phosphorylation of Nck-associated substrates. Finally, the binding (and possibly the phosphorylation) of Nck might serve to relocate these putative effector molecules to a different subcellular compartment. For example, it was recently shown that the membrane localization of Pak1, mediated by the Nck SH3 domain, is sufficient to stimulate its kinase activity and activate downstream mitogen-activated protein kinase cascades [38]. Work is in progress to isolate these Nck-binding phosphoproteins and define their biological activity.

We also demonstrate that the γ 2 isoform of CKI [39] is constitutively associated with Nck in aortic SMC. CKI is a family of second messenger-independent serine/threonine kinases that are expressed ubiquitously in mammalian cells [40]. These enzymes are found in the cytoplasm, in the nucleus and associated with membranes. CKI- γ 2 is the only CKI isoform that contains a proline-rich sequence at its C-terminus [39], which might be responsible for its association with Nck. Interestingly, we found that treatment of aortic SMC with Ang II significantly increased the CKI enzymic activity associated with Nck. However, additional work will be needed to assess whether this change in activity has some biological significance. The exact roles of CKI- γ isoenzymes and the nature of their physiologically relevant substrates still remain undefined. The association of CKI- γ 2 with Nck ([18], and this study) suggests that CKI- γ 2 might phosphorylate Nck directly. In support of this idea, the primary structure of human Nck [3] contains six CKI consensus phosphoacceptor sites [41]. Alternatively, an attractive possibility is that CKI- γ 2 might be involved in the regulation of the AT₁ receptor. CKI has been reported to phosphorylate the insulin receptor [42] and the tumour necrosis factor receptor [43] on serine residues. Most significantly, a recent study showed that CKI- α phosphorylates the m3 muscarinic receptor in an agonist-sensitive manner, suggesting that CKI might provide an alternative route for the regulation of G-protein-coupled receptors [44]. Analysis of the AT₁ receptor sequence reveals the presence of one consensus motif for CKI phosphorylation at the C-terminal extremity and four additional residues that could serve as consensus phosphorylation sites after prior phosphorylation at position -3. Studies are currently under way to determine the subcellular localization of CKI- γ 2 and its ability to phosphorylate Nck and the Ang II AT₁ receptor subtype.

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REFERENCES

- Pawson, T. (1995) *Nature* (London) **373**, 573–580
- Cohen, G. B., Ren, R. and Baltimore, D. (1995) *Cell* **80**, 237–248
- Lehmann, J. M., Riethmuller, G. and Johnson, J. P. (1990) *Nucleic Acids Res.* **18**, 1048
- Li, W., Hu, P., Skolnik, E. Y., Ullrich, A. and Schlessinger, J. (1992) *Mol. Cell. Biol.* **12**, 5824–5833
- Kroll, J. and Wallenberg, J. (1997) *J. Biol. Chem.* **272**, 32521–32527
- Lee, C. H., Li, W., Nishimura, R., Zhou, M., Batzer, A. G., Myers, Jr., M. G., White, M. F., Schlessinger, J. and Skolnik, E. Y. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 11713–11717
- Tang, J. P., Feng, G. S. and Li, W. (1997) *Oncogene* **15**, 1823–1832
- Rivero-Lezcano, O. M., Sameshima, J. H., Marcilla, A. and Robbins, K. C. (1994) *J. Biol. Chem.* **269**, 17363–17366
- Hu, Q., Milfay, D. and Williams, L. T. (1995) *Mol. Cell. Biol.* **15**, 1169–1174
- Rivero-Lezcano, O. M., Marcilla, A., Sameshima, J. H. and Robbins, K. C. (1995) *Mol. Cell. Biol.* **15**, 5725–5731
- Lawe, D. C., Hahn, C. and Wong, A. J. (1997) *Oncogene* **14**, 223–231
- Bagrodia, S., Taylor, S. J., Creasy, C. L., Chernoff, J. and Cerione, R. A. (1995) *J. Biol. Chem.* **270**, 22731–22737
- Galisteo, M. L., Chernoff, J., Su, Y. C., Skolnik, E. Y. and Schlessinger, J. (1996) *J. Biol. Chem.* **271**, 20997–21000
- Bokoch, G. M., Wang, Y., Bohl, B. P., Sells, M. A., Quilliam, L. A. and Knaus, U. G. (1996) *J. Biol. Chem.* **271**, 25746–25749
- Chou, M. M. and Hanafusa, H. (1995) *J. Biol. Chem.* **270**, 7359–7364
- Quilliam, L. A., Lambert, Q. T., Mickelsonyoung, L. A., Westwick, J. K., Sparks, A. B., Kay, B. K., Jenkins, N. A., Gilbert, D. J., Copeland, N. G. and Der, C. J. (1996) *J. Biol. Chem.* **271**, 28772–28776
- Su, Y. C., Han, J. H., Xu, S. C., Cobb, M. and Skolnik, E. Y. (1997) *EMBO J.* **16**, 1279–1290
- Lussier, G. and Larose, L. (1997) *J. Biol. Chem.* **272**, 2688–2694
- Meisenhelder, J. and Hunter, T. (1992) *Mol. Cell. Biol.* **12**, 5843–5856
- Park, D. and Rhee, S. G. (1992) *Mol. Cell. Biol.* **12**, 5816–5823
- Chou, M. M., Fajardo, J. E. and Hanafusa, H. (1992) *Mol. Cell. Biol.* **12**, 5834–5842
- Rockow, S., Tang, J., Xiong, W. and Li, W. (1996) *Oncogene* **12**, 2351–2359
- Garrity, P. A., Rao, Y., Salecker, I., McGlade, J., Pawson, T. and Zipursky, S. L. (1996) *Cell* **85**, 639–650
- Tanaka, M., Lu, W., Gupta, R. and Mayer, B. J. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4493–4498
- Geisterfer, A. A. T., Peach, M. J. and Owens, G. K. (1988) *Circ. Res.* **62**, 749–756
- Berk, B. C., Vekshtein, V., Gordon, H. M. and Tsuda, T. (1989) *Hypertension* **13**, 305–314
- Giasson, E. and Meloche, S. (1995) *J. Biol. Chem.* **270**, 5225–5231
- Dubey, R. K., Jackson, E. K. and Luscher, T. F. (1995) *J. Clin. Invest.* **96**, 141–149
- Catt, K. J., Sandberg, K. and Balla, T. (1993) in *Cellular and Molecular Biology of the Renin–Angiotensin System* (Raizada, M. K., Phillips, M. I. and Summers, C., eds.), pp. 307–356, CRC Press, Boca Raton
- Berk, B. C. and Corson, M. A. (1997) *Circ. Res.* **80**, 607–616
- Lang, U. and Vallotton, M. B. (1989) *Biochem. J.* **259**, 477–483
- Duff, J. L., Berk, B. C. and Corson, M. A. (1992) *Biochem. Biophys. Res. Commun.* **188**, 257–264
- Servant, M. J., Giasson, E. and Meloche, S. (1996) *J. Biol. Chem.* **271**, 16047–16052
- Fleurent, M., Gingras, A. C., Sonenberg, N. and Meloche, S. (1997) *J. Biol. Chem.* **272**, 4006–4012
- Chijiwa, T., Hagiwara, M. and Hidaka, H. (1989) *J. Biol. Chem.* **264**, 4924–4927
- Schmitz, U., Ishida, M. and Berk, B. C. (1997) *Circ. Res.* **81**, 550–557
- Meisenhelder, J., Suh, P. G., Rhee, S. G. and Hunter, T. (1989) *Cell* **57**, 1109–1122
- Lu, W. G., Katz, S., Gupta, R. and Mayer, B. J. (1997) *Curr. Biol.* **7**, 85–94
- Zhai, L., Graves, P. R., Robinson, L. C., Italiano, M., Culbertson, M. R., Rowles, J., Cobb, M. H., DePaoli-Roach, A. A. and Roach, P. J. (1995) *J. Biol. Chem.* **270**, 12717–12724
- Tuazon, P. T. and Traugh, J. A. (1991) *Adv. Second Messenger Phosphoprot. Res.* **23**, 123–164
- Songyang, Z., Lu, K. P., Kwon, Y. T., Tsai, L. H., Filhol, O., Cochet, C., Brickey, D. A., Soderling, T. R., Bartleson, C., Graves, D. J. et al. (1996) *Mol. Cell. Biol.* **16**, 6486–6493
- Rapuano, M. and Rosen, O. M. (1991) *J. Biol. Chem.* **266**, 12902–12907
- Beyaert, R., Vanhaesebroeck, B., Declercq, W., Van Lint, J., Vandenabele, P., Agostinis, P., Vandenheede, J. R. and Fiers, W. (1995) *J. Biol. Chem.* **270**, 23293–23299
- Tobin, A. B., Totty, N. F., Sterlin, A. E. and Nahorski, S. R. (1997) *J. Biol. Chem.* **272**, 20844–20849

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