Tyrosine phosphorylation and association of p130Cas and c-Crk II by ANG II in vascular smooth muscle cells

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Takahashi, Tomosaburo, Yasuhiro Kawahara, Takeshi Taniguchi, and Mitsuhiro Yokoyama. Tyrosine phosphorylation and association of p130Cas and c-Crk II by ANG II in vascular smooth muscle cells. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H1059–H1065, 1998.—In cultured vascular smooth muscle cells (VSMC), angiotensin II (ANG II) stimulated tyrosine phosphorylation of multiple proteins including a 130-kDa protein. This 130-kDa protein was identified as a Crk-associated substrate, p130Cas. ANG II-stimulated tyrosine phosphorylation of p130Cas was rapid, concentration dependent, and inhibited by the AT1-receptor antagonist CV-11974. Neither downregulation of protein kinase C by long exposure of cells to phorbol 12,13-dibutyrate nor blockade of Ca2+ mobilization by 1,2-bis(2-aminophenoxy)-ethane-N,N,N’,N’-tetraacetic acid acetylomethyl ester had an effect on ANG II-stimulated tyrosine phosphorylation of p130Cas. Stimulation with ANG II enhanced the specific association of p130Cas with c-Crk II. The time course of the association of p130Cas and c-Crk II was similar to that of tyrosine phosphorylation of p130Cas. c-Crk II was also tyrosine phosphorylated in response to ANG II. These results indicate that ANG II induces tyrosine phosphorylation of p130Cas and c-Crk II and their specific association, suggesting a potential role of the p130Cas-c-Crk II complex in ANG II signal transduction in VSMC.

AT1 receptor; vascular biology

ANGIOTENSIN II (ANG II), the main peptide hormone of the renin-angiotensin system, has been known to play an important role in the development of various cardiovascular diseases characterized by vascular smooth muscle cell (VSMC) growth, such as hypertension, atherosclerosis, and restenosis after balloon angioplasty, in addition to its key regulatory role in the regulation of blood pressure and circulating volume (8, 31). Although ANG II stimulates hypertrophic growth and migration of VSMC in a cell culture system (8, 31), molecular mechanisms responsible for growth-promoting and cell-migrating actions of ANG II have not been fully understood.

In immunoblot experiments with anti-phosphotyrosine antibody, ANG II induces tyrosine phosphorylation of proteins with apparent molecular masses of 190, 110–130, 70–80, 44, and 42 kDa in VSMC (33). The 44- and 42-kDa proteins have been identified as mitogen-activated protein (MAP) kinase isozymes (32). These isozymes may be involved in the signaling mechanism from the ANG II receptor to the nucleus and play crucial roles in growth-promoting action of ANG II. Recently, 70- to 80-kDa proteins, a part of 110- to 120-kDa proteins, and 190-kDa protein were identified as the focal adhesion-associated protein paxillin, focal adhesion kinase (FAK), and platelet-derived growth factor β-receptor, respectively (16, 17, 23, 34). However, other proteins that are tyrosine phosphorylated in response to ANG II have not been identified.

A Crk-associated substrate (Cas), p130Cas, was originally identified as a protein highly tyrosine phosphorylated in cells transformed by v-src and v-crk oncogenes (12, 19) and forms a stable complex with v-Crk and v-Src in vivo in a phosphorylation-dependent manner (28). Analysis of cDNA for p130Cas reveals that p130Cas contains an Src homology (SH) 3 domain, followed by a "substrate domain" consisting of a cluster of 15 possible SH2 domain-binding sites. p130Cas also has several proline-rich regions that are candidates for SH3 domain-binding sites. These structural characteristics suggest that it may function as an adapter protein in signaling pathways of extracellular ligands. Indeed, several recent reports (1, 22, 24, 35) have shown that cell adhesion to extracellular matrix induces tyrosine phosphorylation of p130Cas, suggesting a role for p130Cas in integrin-mediated signal transduction. However, less is known about the participation of p130Cas in signaling pathways of hormonal ligands, especially ligands for seven-transmembrane G protein–coupled receptors.

In the present study, we first examined whether the 130-kDa protein that was tyrosine phosphorylated in response to ANG II in VSMC was p130Cas. This report shows that the tyrosine phosphorylated 130-kDa protein is indeed p130Cas and that, via ANG II type 1 (AT1) receptors, ANG II stimulates tyrosine phosphorylation of p130Cas in a protein kinase C (PKC)- and Ca2+-independent manner and induces its association with c-Crk II, one of cellular homologs of v-crk oncogene product consisting primarily of SH2 and SH3 domains. Evidence is also provided that c-Crk II is also tyrosine phosphorylated after stimulation with ANG II in VSMC.

MATERIALS AND METHODS

Materials. ANG II, ionomycin, phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-dibutyrate (PDBu), and pertussis toxin were obtained from Sigma (St. Louis, MO). 1,2-Bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid acetylomethyl ester (BAPTA-AM) was from Life Technologies (Rockville, MD). The polyclonal antibodies against p130Cas and c-Src were from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal antibodies against p130Cas, c-Crk II, growth factor receptor-bound protein 2 (GRB2), FAK, a proline-rich tyrosine kinase 2 (PYK2), and phosphotyrosine (PY-20 clone) were from Transduction Laboratories (Lexington, KY). The monoclonal antibody against c-Src was from Upstate Biotechnology (Lake Placid, NY). Protein A Sepharose 4 Fast Flow was from Pharmacia Biotech (Uppsala, Sweden). The AT1-receptor antagonist CV-11974 and the...
AT$_2$-receptor antagonist PD-123319 were gifts from Takeda Pharmaceutical (Osaka, Japan) and Parke-Davis (Ann Arbor, MI), respectively. Other materials and chemicals were obtained from commercial sources.

Cell culture. VSMC were isolated from rat thoracic aorta by enzymatic dissociation as described previously (14). Cells were grown and passaged as described previously (29) and used at passages 6–17.

Preparation of cell lysates and immunoprecipitation. The quiescent VSMC on a 60-mm dish were stimulated with ANG II as indicated. The cells were then washed twice with ice-cold phosphate-buffered saline (PBS) and lysed into either lysis buffer A [PBS, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) containing protease and phosphatase inhibitors (1 mM sodium orthovanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 100 kallikrein-inactivating units/ml of aprotinin)] for p130Cas immunoprecipitation or lysis buffer B (50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5), 1% NP-40, 150 mM NaCl, 5 mM EDTA, and protease and phosphatase inhibitors) for c-Crk II, GRB2, FAK, c-Src, and PYK2 immunoprecipitation. After insoluble materials were removed by centrifugation at 15,000 revolutions/min for 20 min, the protein concentration in the supernatant was normalized using a Bio-Rad protein assay. As whole cell extracts, lysates were directly added 1:4 (vol/vol) to 5× Laemmli sample buffer and boiled at 100°C for 5 min. For immunoprecipitations, the lysates (450µg total protein) were incubated with anti-p130Cas polyclonal antibody (10µg), anti-c-Crk II monoclonal antibody (3µg), anti-GRB2 monoclonal antibody (3µg), anti-FAK monoclonal antibody (3µg), anti-c-Src polyclonal antibody (4µg), or anti-PYK2 monoclonal antibody (3µg) for 2 h at 4°C. Immunocomplexes were immunoprecipitated for 1 h at 4°C with protein A-Sepharose for anti-p130Cas and anti-c-Src polyclonal antibodies, or protein A-Sepharose complexed to rabbit anti-mouse immunoglobulin G (IgG) for the monoclonal antibodies. The immune complexes were washed three times with lysis buffer, and associated proteins were recovered by boiling for 5 min in Laemmli sample buffer.

Immunoblot analysis. Samples were subjected to SDS-polyacrylamide gel electrophoresis (7.5% or 10%), and the separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore). Blots were incubated with the indicated monoclonal antibodies, and primary antibodies were detected using horseradish peroxidase-labeled donkey anti-mouse IgG, followed by enhanced chemiluminescence (Amersham). Stained protein bands were scanned by National Institutes of Health Image software interfaced with a personal computer for quantification.

Statistical analysis. Where applicable, results are expressed as means ± SE. Differences between means were evaluated by t-test where appropriate. A value of $P < 0.05$ was taken to be significant.

RESULTS

Identification of 130-kDa protein that is tyrosine phosphorylated in response to ANG II as p130Cas. As reported previously (33), ANG II stimulation of tyrosine phosphorylation of several proteins in VSMC was assessed by anti-phosphotyrosine immunoblotting of whole cell extracts (Fig. 1A, total lysate). To examine whether the 130-kDa protein that was tyrosine phosphorylated in response to ANG II was related to p130Cas, we performed immunoprecipitation analysis with anti-p130Cas antibody. The tyrosine phosphorylated 130-kDa protein was almost completely immuno-precipitated from the whole cell extracts with anti-p130Cas antibody (Fig. 1A). ANG II caused a striking increase in the tyrosine phosphorylated anti-p130Cas immunoprecipitable band (Fig. 1A). Parallel immunoblots with anti-p130Cas antibody revealed that the recovery of p130Cas from cell lysates was not altered by treatment with ANG II (Fig. 1B). As has been observed in normal fibroblast cells (22, 28), p130Cas was detected as two bands by anti-p130Cas immunoblotting in VSMC. These results indicate that the 130-kDa protein that is tyrosine phosphorylated in response to ANG II is p130Cas.

Kinetic analysis of ANG II-stimulated tyrosine phosphorylation of p130Cas. The effect of ANG II on tyrosine phosphorylation of p130Cas was time and concentration dependent. ANG II-stimulated tyrosine phosphorylation of p130Cas could be detected within 1 min after addition of ANG II, reached a maximum at ~2 min, and then declined rapidly (Fig. 2A). Half-maximal and maximal effects were achieved at 1–10 nM and 100 nM of ANG II, respectively (Fig. 2B).

AT$_1$-receptor-mediated, pertussis toxin-insensitive stimulation of p130Cas tyrosine phosphorylation by ANG II. ANG II receptors have been classified into AT$_1$ and AT$_2$ subtypes (8). ANG II-stimulated tyrosine phosphorylation of p130Cas was completely inhibited by the AT$_1$-
receptor antagonist CV-11974 but not by the AT2 receptor antagonist PD-123319 (Fig. 3). The ANG II receptor has been shown to be linked to a pertussis toxin-sensitive G protein of the Gi subfamily or a pertussis toxin-insensitive G protein of the Gq subfamily in rat aorta (8). Pretreatment with pertussis toxin had no effect on ANG II-stimulated tyrosine phosphorylation of p130Cas (Fig. 3), suggesting that a G protein of the Gq subfamily is involved in this reaction.

PKC- and Ca²⁺-independent stimulation of p130Cas tyrosine phosphorylation by ANG II. In VSMC, AT1-receptor activation is coupled to a phospholipase C-mediated phosphoinositol hydrolysis via a G protein of the Go subfamily, resulting in PKC activation and intracellular Ca²⁺ mobilization (8). We therefore examined whether PKC activation or intracellular Ca²⁺ mobilization was involved in tyrosine phosphorylation of p130Cas. Both the PKC-activating phorbol ester PMA and the Ca²⁺ ionophore ionomycin stimulated tyrosine phosphorylation of p130Cas to almost the same extent as ANG II (Fig. 4). However, neither downregulation of PKC by long exposure of cells to another PKC-activating phorbol ester, PDBu, nor blockade of Ca²⁺ mobilization by the membrane-permeant Ca²⁺ chelator BAPTA-AM had an effect on ANG II-stimulated tyrosine phosphorylation of p130Cas (Fig. 5). Under the same conditions, pretreatment with PDBu inhibited tyrosine phosphorylation of p130Cas stimulated by PMA, but not by ionomycin (Fig. 4), and attenuated ANG II-induced MAP kinase activation (32) and tyrosine phosphorylation of paxillin (data not shown). Pretreatment with BAPTA-AM inhibited tyrosine phosphorylation of p130Cas stimulated by ionomycin, but not by PMA (Fig. 4), and indeed suppressed ANG II-mediated Ca²⁺ mobilization in these cells (data not shown). Thus neither PKC activation nor Ca²⁺ mobilization is involved in ANG II-stimulated tyrosine phosphorylation of p130Cas in VSMC.

Fig. 2. Time course and dose response of ANG II-stimulated tyrosine phosphorylation of p130Cas. Cultured VSMC were treated with 100 nM of ANG II for various periods of time (A) or treated for 2 min with various concentrations of ANG II (B). Anti-p130Cas immunoprecipitates were analyzed by immunoblotting with p-Ty. Blot was stripped and reprobed with anti-p130Cas monoclonal antibody. Tyrosine phosphorylation of p130Cas was quantified by scanning densitometry. Values are means ± SE of 3 independent trials and are percentages of unstimulated levels. Insets: representative blots of 3 independent trials. *P < 0.05 vs. control.

Fig. 3. AT1-receptor-mediated, pertussis toxin-insensitive stimulation of p130Cas tyrosine phosphorylation by ANG II. Cultured VSMC were pretreated with or without 100 nM of CV-11974 for 2 min, 100 nM of PD-123319 for 2 min, or 500 ng/ml of pertussis toxin (PTX) for 5 h. Cells were subsequently treated with or without 100 nM of ANG II for 2 min. A: anti-p130Cas immunoprecipitates were analyzed by immunoblotting with p-Ty. Blot was stripped and reprobed with anti-p130Cas monoclonal antibody. Data represent 1 of 3 independent trials that gave nearly identical results. B: tyrosine phosphorylation of p130Cas was quantified by scanning densitometry. Values are means ± SE of 3 independent trials and are percentages of unstimulated levels. *P < 0.05 vs. respective control.
Stimulation of the specific association of p130Cas and c-Crk II and tyrosine phosphorylation of c-Crk II by ANG II. To examine whether p130Cas associates with other signaling molecules as reported in other cell systems (1, 9, 21, 24, 25, 35), we performed immunoprecipitation analysis with antibodies against either c-Crk II, GRB2, FAK, c-Src, or PYK2. The precipitated proteins were immunoblotted with the anti-p130Cas antibody. Although a small amount of the p130Cas protein was detected in anti-c-Crk II immunoprecipitates in unstimulated VSMC, ANG II induced a marked increase in the amount of p130Cas in the anti-c-Crk II immunoprecipitate (Fig. 6A). The p130Cas protein was not detected in anti-GRB2, anti-FAK, anti-c-Src, or anti-PYK2 immunoprecipitates regardless of stimulation with ANG II (Fig. 6, B–E), indicating that ANG II stimulates the specific interaction between p130Cas and c-Crk II. The formation of the p130Cas-c-Crk II complex was detected within 1 min, reached maximal at ~2 min after stimulation with ANG II, and then declined rapidly (Fig. 7B). This time course was very similar to that of tyrosine phosphorylation of p130Cas. c-Crk II was also tyrosine phosphorylated in response to ANG II (Fig. 7A). The time course of this reaction was slower than those of tyrosine phosphorylation of p130Cas and the formation of the p130Cas-c-Crk II complex, and the maximal level was observed ~5 min after stimulation with ANG II.

DISCUSSION

In the present study, we demonstrated first that the 130-kDa protein that was tyrosine phosphorylated in response to ANG II in VSMC was p130Cas. Using selective AT1- and AT2-receptor antagonists, we further showed that the stimulatory effect of ANG II on tyrosine phosphorylation of p130Cas and c-Crk II was also tyrosine phosphorylated in response to ANG II (Fig. 7A). The time course of this reaction was slower than those of tyrosine phosphorylation of p130Cas and the formation of the p130Cas-c-Crk II complex, and the maximal level was observed ~5 min after stimulation with ANG II.
AT₁-receptor-mediated tyrosine phosphorylation of p130Cas. Via a pertussis toxin-insensitive G protein of the Gq subfamily, activation of AT₁ receptor induces a phospholipase C-mediated phosphoinositide hydrolysis, which causes PKC activation and intracellular Ca²⁺ mobilization (8). Both the PKC-activating phorbol ester PMA and a Ca²⁺-ionophore ionomycin stimulated tyrosine phosphorylation of p130Cas, suggesting possible involvement of PKC and/or Ca²⁺ in ANG II-stimulated tyrosine phosphorylation of p130Cas. However, neither downregulation of PKC by long exposure of cells to PDBu nor chelation of intracellular Ca²⁺ by BAPTA-AM blocked ANG II-stimulated tyrosine phosphorylation of p130Cas, indicating that neither PKC activation nor Ca²⁺ mobilization is involved in ANG II-stimulated tyrosine phosphorylation of p130Cas in VSMC.

FAK is a nonreceptor tyrosine kinase found in focal adhesions (6). Tyrosine phosphorylation of FAK is a rapid response to integrin-mediated cell adhesion and correlates with its kinase activity. p130Cas is also localized primarily in focal adhesions (9, 15) and is tyrosine phosphorylated in response to integrin-mediated cell adhesion (1, 6, 22, 24, 35). In VSMC, FAK is tyrosine phosphorylated in response to ANG II (23). Moreover, FAK was shown to stably associate with p130Cas in BALB/c 3T3 fibroblasts and chicken embryo cells (9, 25). Thus it is possible that FAK is involved in ANG II-stimulated tyrosine phosphorylation of p130Cas. However, we failed to detect the association of p130Cas with FAK in VSMC regardless of stimulation with ANG II. A recent study (35) using fibroblasts deficient in FAK indicated that FAK is not required for tyrosine phosphorylation of p130Cas in response to integrin-mediated cell adhesion. Therefore, FAK seems not to be responsible for ANG II-stimulated tyrosine phosphorylation of p130Cas in VSMC. Other candidates for ANG II-stimulated p130Cas phosphorylation were Src family tyrosine kinases and a new member of the FAK family of nonreceptor tyrosine kinase, PYK2. ANG II was shown to activate c-Src in VSMC (11) and stimulate tyrosine phosphorylation and kinase activity of PYK2 in liver epithelial cells (36). Moreover, Src and PYK2 were shown to associate with p130Cas in fibroblasts and B cells, respectively (1, 21), suggesting potential roles of Src and PYK2 in tyrosine phosphorylation of p130Cas. However, the association of p130Cas with c-Src or PYK2 was not detectable in VSMC. Thus identification of a responsible kinase for ANG II-stimulated tyrosine phosphorylation of p130Cas remains obscure.

In support of a functional significance of p130Cas in ANG II-induced signal transduction, we demonstrated that ANG II treatment significantly enhanced the specific association of p130Cas and c-Crk II. c-Crk II consists of an amino-terminal SH2 domain followed by...
two SH3 domains (20). p130Cas has multiple YD(V/T)P sequences that conform to the binding motif for the Crk-SH2 domain, and c-Crk II was shown to associate with tyrosine phosphorylated p130Cas through its SH2 domain (24, 26, 35). The time course of the association of p130Cas and c-Crk II was similar to that of tyrosine phosphorylation of p130Cas, suggesting that p130Cas binds to c-Crk II in a tyrosine phosphorylation-dependent manner in VSMC. We also demonstrated that c-Crk II was tyrosine phosphorylated in response to ANG II in VSMC. The role of tyrosine phosphorylation of c-Crk II is not clear, but tyrosine phosphorylation of Crk may induce its interaction with other SH2-containing signaling proteins, or perhaps it can produce an intramolecular interaction (7, 27) that may modulate binding activity of the Crk SH3 domain. Through its SH3 domain, Crk has been shown to bind a number of proteins including Sos and C3G, the guanine nucleotide exchange factors for the Ras family of low-molecular-weight guanosine triphosphatases (GTPases) (13, 18, 30). Thus it is possible that tyrosine phosphorylation and association of p130Cas and c-Crk II in response to ANG II may be involved in the activation mechanism of the Ras family of GTPases by recruiting such exchange factors to their vicinities.

p130Cas is a prominent tyrosine phosphorylated protein in cells transformed by several disparate mechanisms (2, 12, 19). Recently, it has been shown (5) that bombesin, a potent mitogen, stimulates p130Cas tyrosine phosphorylation in Swiss 3T3 fibroblasts. Crk was also shown to undergo tyrosine phosphorylation in transformed cells or in response to growth factors (3, 7, 10, 26). Therefore, it is possible that tyrosine phosphorylation of p130Cas and c-Crk II and their specific association may be involved in the cell growth-promoting action of ANG II. In response to integrin-mediated cell adhesion, p130Cas was shown (1, 22, 24, 35) to be tyrosine phosphorylated and associated with c-Crk II. Recently, p130Cas was identified as a substrate of YopH, a bacterial protein tyrosine phosphatase that is translocated into mammalian cells by Yersinia (4). Wild-type YopH promotes detachment of cells from the extracellular matrix, whereas a catalytically inactive form of this protein is defective for cellular detachment. Therefore, it is also possible that ANG II-stimulated tyrosine phosphorylation of p130Cas and formation of p130Cas-c-Crk II complex have a potential role in the processes that regulate cell adhesion and motility.

In summary, the results of the present study provide the first demonstration of potential roles of p130Cas and c-Crk II in ANG II actions in VSMC. Identification of responsible kinases for tyrosine phosphorylation of p130Cas and c-Crk II and downstream elements of p130Cas-c-Crk II complex will be an important issue for understanding their roles in ANG II actions and fully clarifying signaling pathways of ANG II in VSMC.

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