Tyrosine phosphorylation of p62^{Dok} induced by cell adhesion and insulin: possible role in cell migration

Tetsuya Noguchi, Takashi Matozaki^{1,2}, Kenjiro Inagaki, Masahiro Tsuda, Kaoru Fukunaga, Yukari Kitamura, Tadahiro Kitamura, Kozui Shii³, Yuji Yamanashi⁴ and Masato Kasuga

Second Department of Internal Medicine, Kobe University School of Medicine, Kusunoki-cho, Chuo-ku, Kobe 650-0017, ³Hyogo Institute of Clinical Research, 13-70 Kitaohji-cho, Akashi 673-0021,

⁴Department of Oncology, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108-8639, Japan

²Present address: Department of Molecular Biology and Biochemistry, Osaka University Medical School, Suita 565-0871, Japan

¹Corresponding author

e-mail: matozaki@molbio.med.osaka-u.ac.jp

Dok, a 62-kDa Ras GTPase-activating protein (rasGAP)-associated phosphotyrosyl protein, is thought to act as a multiple docking protein downstream of receptor or non-receptor tyrosine kinases. Cell adhesion to extracellular matrix proteins induced marked tyrosine phosphorylation of Dok. This adhesion-dependent phosphorylation of Dok was mediated, at least in part, by Src family tyrosine kinases. The maximal insulin-induced tyrosine phosphorylation of Dok required a Src family kinase. A mutant Dok (Dok Δ PH) that lacked its pleckstrin homology domain failed to undergo tyrosine phosphorylation in response to cell adhesion or insulin. Furthermore, unlike the wild-type protein, Dok Δ PH did not localize to subcellular membrane components. Insulin promoted the association of tyrosine-phosphorylated Dok with the adapter protein NCK and rasGAP. In contrast, a mutant Dok (DokY361F), in which Tyr361 was replaced by phenylalanine, failed to bind NCK but partially retained the ability to bind rasGAP in response to insulin. Overexpression of wild-type Dok, but not that of Dok Δ PH or DokY361F, enhanced the cell migratory response to insulin without affecting insulin activation of mitogen-activated protein kinase. These results identify Dok as a signal transducer that potentially links, through its interaction with NCK and rasGAP, cell adhesion and insulin receptors to the machinery that controls cell motility.

Keywords: cell adhesion/cell migration/Dok/insulin

Introduction

A 62-kDa Ras GTPase-activating protein (rasGAP)-associated phosphotyrosyl protein, now termed Dok (for downstream of kinase), was originally shown to be a common substrate of activated protein tyrosine kinases such as v-Abl, v-Src, v-Fps and v-Fms (Ellis *et al.*, 1990). Tyrosine phosphorylation of Dok correlates well with the trans-

forming activity of such viral oncogene-encoded activated tyrosine kinases, suggesting that this protein plays an important role in cellular transformation (Lugo *et al.*, 1990; Bouton *et al.*, 1991; Moran *et al.*, 1991; DeClue *et al.*, 1993).

Recently, Dok was purified from chronic myelogenous leukemia progenitor cells and from v-Abl-transformed B cells, and its cDNA was cloned (Carpino *et al.*, 1997; Yamanashi and Baltimore, 1997). Other Dok-related proteins, including Dok-2 (Cristofano *et al.*, 1998) and FRIP (Nelms *et al.*, 1998), have also been identified. Dok contains a pleckstrin homology (PH) domain in its N-terminal region; such domains are thought to mediate protein interaction with cellular membranes, possibly by binding to polyphosphoinositides (Musacchio *et al.*, 1993; Lemmon *et al.*, 1996). Dok also contains a putative phosphotyrosine-binding (PTB) domain; such domains are thought to mediate protein–protein interactions by binding to phosphotyrosine (pY)-containing motifs with the sequence NPXpY (Zhou *et al.*, 1995; Eck *et al.*, 1996). In addition, Dok contains several conserved tyrosine phosphorylation motifs that are potential targets for cytosolic tyrosine kinases (Songyang *et al.*, 1995). These tyrosine residues, if phosphorylated, may constitute docking sites for various Src homology 2 (SH2) domain-containing signaling molecules (Songyang *et al.*, 1993).

The overall structure of Dok is thus reminiscent of those of the insulin receptor substrates (IRS) -1 to -4 (Sun *et al.*, 1991, 1995; Lavan *et al.*, 1997a,b) and of Gab1 (Holgado-Madruga *et al.*, 1996), all of which serve as docking proteins for multiple signaling molecules, including the 85-kDa subunit of phosphoinositide (PI) 3-kinase, Grb2, and SHP-2, which act downstream of activated receptors for insulin or epidermal growth factor. Therefore, Dok, like other docking proteins, may be a direct substrate of receptor-type tyrosine kinases. Indeed, Dok undergoes rapid tyrosine phosphorylation in intact cells in response to stimulation of growth factor receptors such as c-Kit and those for epidermal growth factor, insulin-like growth factor-1 and insulin, with their cognate ligands (Bouton *et al.*, 1991; Hosomi *et al.*, 1994; Sanchez-Margalet *et al.*, 1995; Wisniewski *et al.*, 1996). Furthermore, Dok serves as a direct substrate for activated insulin receptors *in vitro* (Hosomi *et al.*, 1994). Thus, Dok may function in signal transduction pathways initiated by a wide range of tyrosine kinases, in both transformed and non-transformed cells. However, the physiological role of tyrosine phosphorylation of Dok is not clear.

In chronic myelogenous leukemia cells expressing p210^{Bcr-Abl}, 293 cells expressing v-Abl, and fibroblasts stimulated with insulin, Dok binds to rasGAP in a tyrosine phosphorylation-dependent manner (Hosomi *et al.*, 1994; Carpino *et al.*, 1997; Yamanashi and Baltimore, 1997). In addition, Dok forms a stable complex with the non-receptor

tyrosine kinase Csk in v-Src-transformed fibroblasts and in B lymphoma cells stimulated with antibodies to immunoglobulin G (Neet and Hunter, 1995; Vuica *et al.*, 1997). Again, however, the physiological significance of the interactions between these proteins and Dok remains to be determined.

Clarification of the mechanism by which tyrosine kinases phosphorylate Dok in intact cells, as well as the identification of signaling proteins that interact with Dok and link Dok phosphorylation to specific cellular responses, should increase our understanding of the biological functions of this protein. We have now shown that cell adhesion to extracellular matrix (ECM) proteins as well as insulin stimulation induce marked tyrosine phosphorylation of Dok, and that this effect requires a Src family kinase. We also show that the PH domain of Dok appears to be required for both tyrosine phosphorylation of the protein and its normal localization to a subcellular membrane component. In addition, phosphorylation of Tyr361 of Dok mediates the association of Dok with the adapter protein NCK in response to insulin. Finally, the overexpression of wild-type Dok enhanced cell migration in response to insulin without affecting insulin-induced activation of mitogen-activated protein (MAP) kinase.

Results

Cell adhesion-induced tyrosine phosphorylation of Dok mediated by a Src family kinase

We observed that tyrosine phosphorylation of Dok remained apparent in cells deprived of serum for up to 24 h (data not shown), prompting us to examine the mechanism by which phosphorylation of Dok is regulated under such conditions. Several proteins that accumulate at focal contacts, such as focal adhesion kinase (Fak), tensin, paxillin and p130^{Cas}, undergo tyrosine phosphorylation in a cell adhesion-dependent manner (Burrige *et al.*, 1992; Bockholt and Burrige, 1993; Nojima *et al.*, 1995; Hanks and Polte, 1997). We therefore investigated whether cell adhesion to ECM proteins regulates tyrosine phosphorylation of Dok. Chinese hamster ovary (CHO) cells that overexpress human insulin receptors (CHO-IR cells) or v-Src-transformed 3Y1 (SR-3Y1) fibroblasts were deprived of serum for 12 h and then incubated in phosphate-buffered saline (PBS) in the absence or presence of 1 mM Ca²⁺ for 0–15 min (Figure 1A). Treatment of cells with PBS in the absence of Ca²⁺ partially disrupts cell adhesion to the substratum, resulting in a change in cell morphology from a flattened spindle shape to spherical (Fujioka *et al.*, 1996). Whole-cell lysates were prepared from the treated cells, after which Dok was subjected to immunoprecipitation with the specific monoclonal antibody (mAb) 2C4, followed by immunoblot analysis with mAb PY20 to phosphotyrosine. In CHO-IR cells, the extent of tyrosine phosphorylation of Dok decreased markedly in a time-dependent manner during incubation with PBS in the absence of Ca²⁺ (Figure 1A). The presence of Ca²⁺ during incubation prevented this decrease in Dok phosphorylation. In contrast, the extent of tyrosine phosphorylation of Dok in SR-3Y1 cells was much greater than that apparent in CHO-IR cells, and it was not substantially affected by incubation of the cells in the absence of Ca²⁺ (Figure 1A), suggesting that constitutive

activation of Src may compensate for the dephosphorylation of Dok induced by the removal of Ca²⁺.

Because Ca²⁺ removal might disrupt cell–cell adhesion as well as cell–substratum adhesion, these experiments could not distinguish which type of adhesion was affecting the tyrosine phosphorylation of Dok. This issue was addressed by immunoprecipitating Dok from lysates of serum-deprived CHO-IR cells that had been cultured to a confluency of 100 or 30%; at the high density, all cells adhered to adjacent cells, whereas at the low density they did not. The extent of tyrosine phosphorylation of Dok was not affected by cell density (Figure 1B), suggesting that cell–cell adhesion does not regulate the tyrosine phosphorylation of this protein.

To examine further the effect of cell adhesion on the tyrosine phosphorylation of Dok, we detached serum-deprived CHO-IR cells from the culture dish and then either maintained them in suspension or replated them on culture dishes that had been coated with various ECM proteins (Figure 1C). In addition, tyrosine phosphorylation of Fak, which is regulated by an integrin-mediated pathway (Yamada and Miyamoto, 1995; Hanks and Polte, 1997), was also examined. The extent of tyrosine phosphorylation of Fak increased markedly in cells cultured on fibronectin or laminin, but not in those cultured on bovine serum albumin (BSA) (Figure 1C). Adhesion of cells to poly-L-lysine, a non-specific substrate, also increased the phosphotyrosine content of Fak. The extent of tyrosine phosphorylation of Dok was markedly increased by cell adhesion to poly-L-lysine or fibronectin; Dok phosphorylation was increased to a lesser extent in cells cultured in BSA-coated dishes (Figure 1C). The extent of tyrosine phosphorylation of Dok in cells cultured on poly-L-lysine was substantially greater than that apparent in cells cultured on fibronectin; this was not the case with Fak. Furthermore, adhesion to laminin-coated dishes, which markedly increased phosphorylation of Fak, induced only a small increase in tyrosine phosphorylation of Dok (Figure 1C). Adhesion of CHO-K1 cells, which did not overexpress insulin receptors, to fibronectin or poly-L-lysine also markedly increased tyrosine phosphorylation of Dok (Figure 1D), indicating that adhesion-dependent tyrosine phosphorylation of Dok was not caused by overexpression of insulin receptors. In addition, the extent of tyrosine phosphorylation of Dok in CHO-K1 cells cultured on poly-L-lysine was again substantially greater than that apparent in cells cultured on fibronectin (Figure 1D). These results indicate that cell adhesion to ECM proteins stimulates tyrosine phosphorylation of Dok, and that this effect is mediated by a mechanism that differs from that responsible for tyrosine phosphorylation of Fak.

Cell adhesion to ECM proteins such as fibronectin induces the activation of Src kinase (Cobb *et al.*, 1994; Schlaepfer *et al.*, 1994). In addition, Dok is a substrate for tyrosine phosphorylation by v-Src (Ogawa *et al.*, 1994), suggesting that Src or another Src family kinase, such as Fyn, might be responsible for the tyrosine phosphorylation of Dok in response to cell adhesion. To investigate this hypothesis, we examined the effects of overexpression of wild-type Csk or a kinase-inactive mutant of Csk on tyrosine phosphorylation of Dok. Csk is a Src-like tyrosine kinase that inhibits the activity of

Fig. 1. Effects of cell-substratum adhesion (A), cell-cell adhesion (B), and cell adhesion to various ECM proteins (C and D) on tyrosine phosphorylation of Dok. (A) CHO-IR cells and SR-3Y1 fibroblasts were deprived of serum for 12 h and subsequently incubated in PBS in the absence or presence of 1 mM CaCl_2 for 0–15 min as indicated. Whole-cell lysates were then subjected to immunoprecipitation (IP) with mAb 2C4 to Dok, and the resulting immunoprecipitates were subjected to immunoblot analysis with either horseradish peroxidase (HRP)-conjugated mAb PY20 to phosphotyrosine (α PY) (upper panel) or polyclonal antibodies to Dok (α Dok) (lower panel). The positions of Dok and tyrosine-phosphorylated Dok (Dok-P) are indicated. (B) Lysates prepared from serum-deprived CHO-IR cells cultured to a confluency of 100% (high density) or 30% (low density) were subjected to immunoprecipitation with mAb 2C4 and subsequent immunoblot analysis with either HRP-conjugated mAb PY20 (upper panel) or polyclonal antibodies to Dok (lower panel). (C) Serum-deprived CHO-IR cells were detached from the culture dish, either maintained in suspension (Susp) or replated onto culture dishes coated with the indicated proteins, and then incubated for 1 h. Cell lysates were then subjected to immunoprecipitation with either mAb 2C4 (upper panel) or polyclonal antibodies to Fak (α Fak) (lower panel), and the resulting immunoprecipitates were subjected to immunoblot analysis with HRP-conjugated mAb PY20. The same immunoprecipitates were also subjected to immunoblot analysis with polyclonal antibodies to Dok or to Fak to ensure that equal amounts of the respective protein were present in each lane. (D) Serum-deprived CHO-K1 cells were detached from the culture dish, and then maintained in suspension (Susp) or replated onto culture dishes coated with the indicated proteins. Tyrosine phosphorylation of Dok was determined as described in legend for (C).

Src family kinases by catalyzing the phosphorylation of a C-terminal tyrosine residue (Nada *et al.*, 1991; Okada *et al.*, 1991). Lysates were prepared from CHO-IR cells and from CHO-IR cells that overexpress wild-type Csk (CHO-Csk-WT cells) or a catalytically inactive Csk (CHO-Csk-K/R cells). In CHO-Csk-K/R cells, endogenous Src family kinases are activated (Tsuda *et al.*, 1998). In contrast, they are expected to be inactivated in CHO-Csk-WT cells. Dok was immunoprecipitated from the lysates with mAb 2C4 and then subjected to immunoblot analysis with mAb PY20. The amount of phosphotyrosine in Dok immunoprecipitated from CHO-Csk-K/R cells was

markedly increased compared with that in the protein precipitated from the parental cells (Figure 2A). The presence of equal amounts of Dok in the immunoprecipitates from the two cell types was confirmed by immunoblot analysis with polyclonal antibodies to Dok. This result is consistent with the notion that Dok is a good substrate for endogenous Src family kinases. In contrast, overexpression of wild-type Csk slightly increased tyrosine phosphorylation of Dok compared with that of the parental cells (Figure 2A). It is possible that highly overexpressed Csk might directly tyrosine phosphorylate Dok and result in a small increase in overall tyrosine phosphorylation of

Fig. 2. Tyrosine phosphorylation of Dok in cells overexpressing wild-type Csk or a catalytically inactive Csk (A) or in cells lacking Src or Fyn (B). (A) Lysates prepared from parental CHO-IR cells, CHO-Csk-WT cells or from CHO-Csk-K/R cells were subjected to immunoprecipitation with mAb 2C4 to Dok, and the resulting immunoprecipitates were subjected to immunoblot analysis with either HRP-conjugated mAb PY20 (upper panel) or polyclonal antibodies to Dok (lower panel). (B) Cells that lack Src (Src^{-/-}) or Fyn (Fyn^{-/-}) and the corresponding wild-type (WT) cells were serum-deprived, detached from the culture dish and then maintained in suspension (Susp) or replated onto culture dishes coated with the indicated proteins. Cell lysates were then subjected to immunoprecipitation with mAb 2C4 and subsequent immunoblot analysis with either HRP-conjugated mAb PY20 (upper panel) or polyclonal antibodies to Dok (lower panel).

Dok, even though endogenous Src family kinases were inactivated.

To determine which of the Src family kinases mediate adhesion-dependent tyrosine phosphorylation of Dok, we analyzed Dok phosphorylation in cells derived from Src or Fyn knockout mice. When Src^{-/-} cells were replated onto culture dishes coated with fibronectin or poly-L-lysine, the amounts of phosphotyrosine in Dok immunoprecipitated from Src^{-/-} cells were substantially reduced relative to those in the protein isolated from wild-type cells (Figure 2B). In contrast, the extent of tyrosine phosphorylation of Dok induced by cell adhesion in Fyn^{-/-} cells was almost the same as that of Dok in wild-type cells (Figure 2B). These results indicate that adhesion-dependent tyrosine phosphorylation of Dok is regulated by Src but not by Fyn. However, given that a substantial amount of phosphotyrosine was present in Dok precipitated from Src^{-/-} cells, it is probable that Src family kinases other than Src, or members of another family of tyrosine kinases, also contribute to Dok phosphorylation.

Synergistic effects of insulin and cell-substratum adhesion on tyrosine phosphorylation of Dok

Activated insulin receptors mediate the tyrosine phosphorylation of Dok both *in vitro* and *in vivo* (Hosomi *et al.*, 1994). We therefore examined whether cell-substratum adhesion or Src family kinases are required for insulin-induced Dok phosphorylation. Consistent with previous observations (Hosomi *et al.*, 1994), insulin markedly increased the extent of tyrosine phosphorylation of Dok in adherent CHO-IR cells (Figure 3A). Tyrosine phosphorylation of Dok was almost undetectable after cells were detached from the culture dish and maintained in suspension. Insulin also increased the tyrosine phosphorylation of Dok in the suspended cells, although to a lesser extent than that apparent in adherent cells (Figure 3A). We next pre-incubated serum-deprived CHO-IR cells in the absence or presence of PP1, a specific inhibitor of Src family kinases (Daub *et al.*, 1997), and then examined the effect of insulin stimulation. PP1 almost completely abolished the tyrosine phosphorylation of Dok in adherent cells (Figure 3B). Although insulin increased the tyrosine phosphorylation of Dok in cells treated with PP1, the effect was less marked than that apparent in control cells. These results indicate that optimal tyrosine phosphorylation of Dok in response to insulin requires cell adhesion and a Src family kinase.

Phosphorylation of a juxtamembrane tyrosine residue (Tyr960) of the insulin receptor is required for insulin-induced tyrosine phosphorylation of endogenous substrates such as IRS-1 and Shc (White *et al.*, 1988; Yonezawa *et al.*, 1994). We therefore examined whether Tyr960 of the insulin receptor is also required for insulin-induced tyrosine phosphorylation of Dok. CHO-IR cells or CHO cells that overexpress mutant insulin receptors in which Tyr960 is replaced by alanine (CHO-IRY960A cells) were transiently transfected with cDNA encoding a hemagglutinin (HA) epitope-tagged form of Dok. Insulin induced tyrosine phosphorylation of Dok in CHO-IR cells but not in CHO-IRY960A cells (Figure 3C), indicating that Tyr960 of the insulin receptor is required for Dok phosphorylation in response to insulin.

Role of the PH domain of Dok in its tyrosine phosphorylation and subcellular localization

The PH domain of IRS-1 is required for tyrosine phosphorylation of the protein in response to insulin (Myers *et al.*, 1995; Voliovitch *et al.*, 1995). Given that the overall structure of Dok is similar to that of IRS-1, we hypothesized that the PH domain of Dok might also be required for phosphorylation of this protein by the insulin receptor kinase. To test this hypothesis, we generated a cDNA encoding a truncated form of Dok (Dok Δ PH) that lacks 80 amino acids corresponding to the PH domain. We then prepared CHO-IR cell lines that stably overexpress wild-type Dok (CHO-DokWT cells) or the truncated Dok (CHO-Dok Δ PH cells). Immunoblot analysis with antibodies to Dok revealed that the amounts of exogenous Dok proteins expressed in the two cell lines were similar (Figure 7A).

CHO-DokWT and CHO-Dok Δ PH cells were incubated in the absence or presence of insulin, and the extent of tyrosine phosphorylation of each Dok protein was determined. Tyrosine phosphorylation of DokWT was

Fig. 3. Effects of cell detachment from the substratum (A), PP1 pre-treatment (B), or substitution of Tyr960 of the insulin receptor with alanine (C) on insulin-induced tyrosine phosphorylation of Dok. (A) CHO-IR cells were deprived of serum for 12 h, either maintained attached to (On dish) or detached from (Off dish) the culture dish, and subsequently incubated for 5 min in the absence or presence of 100 nM insulin. Whole-cell lysates were then subjected to immunoprecipitation with mAb 2C4 to Dok, and the resulting immunoprecipitates were subjected to immunoblot analysis with either HRP-conjugated mAb PY20 (upper panel) or polyclonal antibodies to Dok (lower panel). (B) Serum-deprived CHO-IR cells were incubated for 30 min with or without 10 μ M PP1, and then for 5 min in the absence or presence of 100 nM insulin. Cell lysates were subjected to immunoprecipitation with mAb 2C4, followed by immunoblot analysis with either HRP-conjugated mAb PY20 (upper panel) or polyclonal antibodies to Dok (lower panel). (C) CHO-IR or CHO-IRY960A cells were transiently transfected with a vector encoding HA epitope-tagged Dok, deprived of serum, and then incubated for 5 min in the absence or presence of 100 nM insulin. Cell lysates were subjected to immunoprecipitation with mAb 12CA5 to the HA epitope (α HA), followed by immunoblot analysis with either HRP-conjugated mAb PY20 (upper panel) or polyclonal antibodies to Dok (lower panel).

apparent in unstimulated cells, and the extent of phosphorylation was further increased in response to insulin stimulation (Figure 4A). However, tyrosine phosphoryl-

Fig. 4. Tyrosine phosphorylation (A) and subcellular localization (B) of a mutant Dok lacking an intact PH domain. (A) CHO-DokWT and CHO-Dok Δ PH cells were deprived of serum and subsequently incubated for 5 min in the absence or presence of 100 nM insulin. Whole-cell lysates were then subjected to immunoprecipitation with mAb 12CA5 to the HA epitope, followed by immunoblot analysis with either HRP-conjugated mAb PY20 (upper panel) or polyclonal antibodies to Dok (lower panel). (B) CHO-DokWT and CHO-Dok Δ PH cells were fractionated into nuclear (Nuc), cytosolic (Cyt), membrane (Mem), cytoskeletal (C-SK), and membrane-skeleton (M-SK) components as described in Materials and methods. Each fraction was then subjected to immunoblot analysis with the anti-HA epitope mAb 12CA5. The immunoreactive material apparent immediately below Dok Δ PH in the lanes for M-SK in (B) comprises non-specific cross-reactive polypeptides of unknown origin.

ation of Dok Δ PH was not evident in either unstimulated or stimulated cells (Figure 4A). The amounts of the two Dok proteins in the respective immunoprecipitates were almost identical, as revealed by immunoblot analysis with antibodies to Dok (Figure 4A). In addition, cell adhesion to ECM proteins did not induce tyrosine phosphorylation of Dok Δ PH (data not shown). These results suggest that an intact PH domain is required for tyrosine phosphorylation of Dok in both unstimulated and insulin-stimulated adherent cells.

We also examined the effect of PH domain deletion on the subcellular localization of Dok. Subcellular fractions of the transfected cells were subjected to immunoblot analysis with mAb 12CA5 to the HA epitope. A substantial amount of DokWT was detected in all fractions with the exception of the cytoskeletal fraction (Figure 4B). In contrast, Dok Δ PH was not detected in the membrane fraction (Figure 4B). In addition, we detected only a small amount of Dok Δ PH in the membrane-skeleton fraction, although substantial amounts of the protein were apparent in the nuclear and cytosolic fractions (Figure 4B). These

results suggest that an intact PH domain is required for normal localization of Dok to subcellular components, especially to cellular membranes and the membrane skeleton.

Binding of NCK to tyrosine-phosphorylated Dok through the Y361DEP motif

Tyrosine-phosphorylated Dok binds the SH2 domain of rasGAP (Yamanashi and Baltimore, 1997). We therefore examined the effects of cell adhesion and insulin on the interaction of Dok with SH2 domain-containing signaling molecules. Various candidate proteins were thus immunoprecipitated with specific antibodies from insulin-stimulated CHO-DokWT cells, and the resulting precipitates were examined by immunoblot analysis with mAb PY20 to phosphotyrosine (Figure 5A). As demonstrated previously (Ellis *et al.*, 1990), a 60-kDa phosphotyrosine-containing protein, which co-migrated with Dok, was co-immunoprecipitated with rasGAP. Of the various other immunoprecipitates analyzed, a similar phosphoprotein was present only in that prepared with antibodies to NCK (Figure 5A), suggesting that NCK may associate with tyrosine-phosphorylated Dok. We then subjected immunoprecipitates prepared from unstimulated adherent CHO-DokWT cells with a mAb to NCK to immunoblot analysis with antibodies to Dok, revealing that Dok was indeed co-immunoprecipitated with NCK (Figure 5B). Treatment of adherent cells with PBS in the absence of Ca^{2+} reduced, whereas insulin stimulation increased the amount of Dok associated with NCK (Figure 5B). These observations indicate that NCK binds to tyrosine-phosphorylated Dok in response to cell adhesion or insulin.

In vitro binding studies with a phosphotyrosyl peptide library have shown that the SH2 domain of NCK binds preferentially to phosphopeptides that contain the sequence motif pYDEP (Songyang *et al.*, 1993). Given that Dok contains the sequence Y361DEP, we investigated whether this sequence mediates the binding of NCK to Dok. We generated a CHO-IR cell line that stably overexpresses a mutant Dok in which Tyr361 is replaced by phenylalanine (CHO-DokY361F cells). The amount of total Dok protein expressed in these cells was similar to that present in CHO-DokWT cells (see Figure 7A). CHO-DokWT and CHO-DokY361F cells were incubated in the absence or presence of insulin, after which the extent of tyrosine phosphorylation of wild-type or mutant Dok as well as the association of these proteins with rasGAP and NCK were examined (Figure 6). Tyrosine phosphorylation of the mutant DokY361F in unstimulated adherent cells was almost completely abolished compared with that of wild-type Dok (Figure 6). In contrast, insulin effectively induced the phosphorylation of the mutant protein. The amounts of rasGAP and NCK co-immunoprecipitated with wild-type Dok were substantially increased by treatment of cells with insulin (Figure 6). In contrast, NCK did not bind to the mutant Dok in the absence or presence of insulin stimulation. Although insulin induced the association of DokY361F with rasGAP, this effect was less marked than that on the interaction of rasGAP with DokWT (Figure 6). These results further confirmed that NCK associates with Dok in response to insulin, and they showed that Tyr361 of Dok is required for this interaction. Furthermore, this tyrosine residue might participate in formation of the

Fig. 5. Association of NCK with tyrosine-phosphorylated Dok. (A) Interaction of various SH2 domain-containing signaling molecules with phosphotyrosyl proteins in insulin-stimulated CHO-DokWT cells. Serum-deprived cells were incubated for 5 min with 100 nM insulin, after which cell lysates were subjected to immunoprecipitation with mAb 12CA5 to the HA epitope, mAb B4F8 to rasGAP (α rasGAP), or mAb 6C3 to NCK (α NCK), or with polyclonal antibodies to Grb2 (α Grb2), Csk (α Csk), SHP-2 (α SHP-2) or c-Abl (α c-Abl). The resulting immunoprecipitates were then subjected to immunoblot analysis with HRP-conjugated mAb PY20. Whole-cell lysate (WCL) was also directly subjected to immunoblot analysis with PY20. The positions of IRS-1, SHPS-1, the insulin receptor (IR), Dok and Shc are indicated, as are those of molecular size standards (in kilodaltons). (B) Effects of insulin stimulation and partial disruption of cell-substratum adhesion on the association of NCK with Dok. Serum-deprived CHO-DokWT cells were incubated with or without 100 nM insulin for 5 min, or with PBS in the absence of Ca^{2+} for 15 min. Cell lysates were then subjected to immunoprecipitation with mAb 6C3 to NCK, followed by immunoblot analysis with either HRP-conjugated mAb PY20 (top panel), polyclonal antibodies to Dok (middle panel), or mAb to NCK (α NCK) (bottom panel).

Dok-rasGAP complex, although we cannot exclude the possibility that substitution of this residue results in a structural alteration of the Dok protein that inhibits binding of rasGAP.

Effects of overexpression of Dok on insulin-induced activation of MAP kinase

We investigated the functional consequences of the overexpression of wild-type or mutant Dok proteins. The

Fig. 6. Effects of the substitution of Tyr361 of Dok with phenylalanine on tyrosine phosphorylation of Dok and on its association with rasGAP and NCK in response to insulin. CHO-DokWT or CHO-DokY361F cells were deprived of serum and then incubated for 5 min in the absence or presence of 100 nM insulin. Whole-cell lysates were subjected to immunoprecipitation with mAb 12CA5 to the HA epitope, followed by immunoblot analysis with either mAb B4F8 to rasGAP (top panel), HRP-conjugated mAb PY20 (second panel), polyclonal antibodies to Dok (third panel), or a mAb to NCK (bottom panel).

amounts of total Dok protein in CHO-DokWT, CHO-Dok Δ PH and CHO-DokY361F cells were almost identical and ~ 10 times that of endogenous Dok protein in parental CHO-IR cells (Figure 7A). It was possible that overexpression of Dok would affect the activity or subcellular localization of rasGAP, and thereby alter the activation state of Ras. If so, insulin-induced activation of MAP kinase, which is mediated in a Ras-dependent manner (de Vries-Smits *et al.*, 1992), might also be affected. Immunoblot analysis of the membrane fraction of CHO-IR cells revealed that insulin induced a small increase in the membrane association of rasGAP (Figure 7B). The overexpression of wild-type Dok markedly increased the amount of rasGAP associated with the membrane fraction of cells stimulated with insulin (Figure 7B). Nevertheless, we did not detect a substantial difference in the extent of MAP kinase activation between CHO-IR cells and CHO-DokWT cells exposed to various concentrations of insulin (Figure 7C and D). Thus, formation of a complex between Dok and rasGAP appears not to affect Ras or subsequent MAP kinase activation in response to insulin.

Effects of overexpression of Dok or Csk on insulin-stimulated cell motility

Cell migration is controlled by a complex mechanism that includes reorganization of the actin-based cytoskeleton and adhesion to ECM proteins (Mithison and Cramer, 1996). Given that insulin stimulates cell motility (Yenush *et al.*, 1994) and that tyrosine phosphorylation of Dok is regulated by cell adhesion and insulin, it was possible that overexpression of Dok might affect insulin stimulation of

cell migration on ECM protein. To test this hypothesis, we assayed the abilities of the transfected cells to migrate on a fibronectin-coated membrane toward an insulin source with the use of a Boyden chamber. Insulin induced an ~ 2.2 -fold increase in the migration rate of parental CHO-IR cells (Figure 8A). The basal migration rate of CHO-DokWT cells was ~ 1.6 times that of the parental cells, whereas in the presence of insulin the migration rate of CHO-DokWT cells was further increased to a value ~ 3.5 times that of the basal rate for CHO-IR cells (Figure 8A). Thus, the overexpression of wild-type Dok enhanced the ability of insulin to stimulate cell migration on fibronectin. In contrast, the rates of migration of CHO-Dok Δ PH cells and CHO-DokY361F cells did not differ substantially from that of parental cells under either basal or stimulated conditions (Figure 8A). These results suggest that Dok may contribute to the mechanism by which insulin stimulates cell motility on fibronectin, and that this process may require both the PH domain of Dok and phosphorylation of Dok on Tyr361.

We also assayed the abilities of CHO-Csk-WT cells and CHO-Csk-K/R cells to migrate on a fibronectin-coated membrane toward an insulin source. The basal migration rate of CHO-Csk-K/R cells was ~ 2.3 times that of the parental cells (Figure 8B). Insulin stimulation further increased the migration rate of CHO-Csk-K/R cells to a value ~ 4.6 times that of the basal rate for CHO-IR cells (Figure 8B). Thus, the overexpression of a kinase-inactive mutant of Csk markedly enhanced the ability of insulin to stimulate cell migration on fibronectin. In contrast, the rate of migration of CHO-Csk-WT cells did not differ significantly from that of parental cells under either basal or stimulated conditions (Figure 8B). Given that the extent of tyrosine phosphorylation of Dok was markedly increased in CHO-Csk-K/R cells (Figure 2A), these results suggest that the migratory response of CHO-IR cells may correlate well with tyrosine phosphorylation of Dok.

Finally, we examined the effect of treatment of cells with PP1 on cell migration. Treatment with PP1 markedly decreased the migration rate of CHO-IR cells to a value ~ 0.27 times that of the rate for untreated CHO-IR cells under unstimulated conditions (Figure 8C). Although insulin increased the migratory response of cells treated with PP1, the effect was less marked than that apparent in untreated cells (~ 1.4 - versus ~ 2.7 -fold over unstimulated CHO-IR cells) (Figure 8C). These results again indicate that cell migratory response correlates with tyrosine phosphorylation of Dok (see Figure 3B), supporting our notion that Dok may contribute to the mechanism by which insulin stimulates cell motility on fibronectin.

Discussion

We have shown that cell adhesion to ECM proteins induces tyrosine phosphorylation of Dok. Such adhesion has previously been shown to induce autophosphorylation of Fak by a mechanism that involves the engagement of integrins by ECM proteins such as fibronectin and laminin (Yamada and Miyamoto, 1995; Hanks and Polte, 1997). However, tyrosine phosphorylation of Dok was markedly increased by cell adhesion to poly-L-lysine as well as to fibronectin, but not by adhesion to laminin. Thus, the mechanism by which cell adhesion induces tyrosine phosphorylation of

Fig. 7. Effects of overexpression of Dok on insulin-induced membrane association of rasGAP and activation of MAP kinase. (A) Expression of Dok proteins in CHO-IR, CHO-DokWT, CHO-DokY361F and CHO-DokΔPH cells was assessed by subjecting cell lysates to immunoblot analysis with polyclonal antibodies to Dok. (B) CHO-IR and CHO-DokWT cells were deprived of serum and then incubated for 5 min with or without 100 nM insulin. A solubilized membrane fraction was prepared as described in Materials and methods, and was subjected to immunoblot analysis with mAb B4F8 to rasGAP. (C) Serum-deprived CHO-IR and CHO-DokWT cells were incubated for 5 min with the indicated concentrations of insulin, after which whole-cell lysates were prepared and subjected to immunoblot analysis with antibodies specific for tyrosine-phosphorylated (activated) MAP kinase (αMAPK-P) (upper panel). The same blot was also probed with α91 polyclonal antibodies to p44 and p42 isoforms of MAP kinase to confirm that similar amounts of the enzyme were present in each lane (lower panel). (D) The extent of tyrosine phosphorylation of p44 MAP kinase in (C) was quantified by scanning densitometry with the NIH image program. The data are expressed as a percentage of the value for parental CHO-IR cells exposed to 10 nM insulin.

Dok may differ from that by which it increases tyrosine phosphorylation of Fak.

With regard to the mechanism by which cell adhesion induces Dok phosphorylation, we showed that the overexpression of a kinase-inactive Csk mutant markedly increased the tyrosine phosphorylation of Dok in adherent cells, compared with that apparent in parental cells. Csk inhibits the activities of Src family kinases by phosphorylating a C-terminal tyrosine residue in these enzymes (Nada *et al.*, 1991; Okada *et al.*, 1991). Thus, overexpression of the Csk mutant may increase the tyrosine phosphorylation of Dok by competitively inhibiting the action of endogenous Csk and thereby increasing the activities of Src family kinases. In addition, targeted disruption of the *Src* gene or pharmacological inhibition of Src family kinases resulted in a decrease in the extent of cell adhesion-induced tyrosine phosphorylation of Dok. Thus, cell adhesion-dependent tyrosine phosphorylation of Dok may be mediated, at least in part, by endogenous Src family kinases. Cell adhesion to ECM proteins has previously been shown to increase the extent of tyrosine phosphorylation of various proteins, including paxillin (Bockholt and Burridge, 1993), SHPS-1 (Tsuda *et al.*, 1998) and p130^{Cas}

(Nojima *et al.*, 1995) in addition to Fak (Hanks and Polte, 1997), all of which are thought to be substrates for Src family kinases and localize at focal adhesion contacts. Thus, Dok might be a new member of the group of proteins that are localized at focal adhesion contacts and undergo tyrosine phosphorylation in response to the interaction of cells with the ECM.

Activated insulin receptors have previously been shown to catalyze the tyrosine phosphorylation of Dok (Hosomi *et al.*, 1994). We have now shown that insulin induced the tyrosine phosphorylation of Dok in cells that had been detached from the culture dish or in which endogenous Src family kinases were inhibited. Thus, insulin stimulation of the tyrosine phosphorylation of Dok requires neither cell-substratum adhesion nor Src family kinases in CHO-IR cells. However, the extent of tyrosine phosphorylation of Dok in insulin-stimulated adherent cells was substantially greater than that apparent in insulin-stimulated suspended cells, indicating that maximal tyrosine phosphorylation of Dok requires both cell adhesion and insulin stimulation. We also showed that insulin fails to induce tyrosine phosphorylation of Dok in CHO cells overexpressing a mutant insulin receptor in which the putative

Fig. 8. Effects of overexpression of Dok (A) or Csk (B) and PP1 treatment (C) on insulin-induced cell migration on fibronectin. (A and B) Parental CHO-IR cells and transfected cells overexpressing the indicated Dok proteins (A) or Csk proteins (B) were assayed for migration through a fibronectin-coated filter toward a chamber containing or not containing 5 nM insulin as described in Materials and methods. (C) CHO-IR cells treated with or without 10 μ M PP1 were assayed for migration as described in Materials and methods. The number of cells that had migrated was counted and expressed as a percentage of the control value for unstimulated parental CHO-IR cells. Data are means $\pm$ SE of three independent experiments.

autophosphorylation site at Tyr960 was changed to alanine. This observation is consistent with previous data showing a requirement for Tyr960 of the insulin receptor for insulin-induced tyrosine phosphorylation of a 60-kDa rasGAP-associated protein (Danielsen and Roth, 1996). The juxtamembrane Tyr960 residue is part of an NPXY960 motif that mediates the interaction of activated insulin receptors with PTB domains of IRS-1 and Shc (He *et al.*, 1995; Wolf *et al.*, 1995). Given that Dok also contains a putative PTB domain (Carpino *et al.*, 1997; Yamanashi and Baltimore, 1997), it is probable that Dok also associates through this domain with the NPXY960 motif of activated insulin receptors and then undergoes tyrosine phosphorylation.

We have also shown that the PH domain of Dok is essential for Dok phosphorylation induced not only by cell adhesion, but also by insulin. The PH domain is thought to mediate the interaction between signaling molecules containing it, and cellular membranes, presumably through its binding to polyphosphoinositides (Musacchio *et al.*, 1993; Lemmon *et al.*, 1996). Indeed, we showed by subcellular fractionation that wild-type Dok was present in the membrane fraction as well as in the nuclear, cytosolic and membrane-skeleton fractions of adherent cells. In contrast, a mutant Dok lacking an intact PH domain failed to localize to cellular membranes. Thus, through the binding of its PH domain to polyphosphoinositides, Dok may localize to the cell membrane, where insulin receptors and Src family kinases are also located; such localization may be a prerequisite for the tyrosine phosphorylation of Dok by the insulin receptor kinase or Src family kinases. Alternatively, Dok might directly associate with insulin receptors or Src family kinases through its PH domain. Consistent with this latter possibility, IRS-1 interacts with activated insulin receptors through its PH domain (Myers *et al.*, 1995; Voliovitch *et al.*, 1995), although the PH domain of Dok does not show marked sequence homology to that of IRS-1 (Carpino *et al.*, 1997; Yamanashi and Baltimore, 1997).

To shed light on the physiological roles of Dok, we investigated which SH2 domain-containing proteins bind to tyrosine-phosphorylated Dok in response to cell adhesion or insulin. Of the various SH2 domain-containing molecules examined, we found that tyrosine-phosphorylated Dok binds to NCK as well as to rasGAP in response to these stimuli. Furthermore, mutation of Tyr361 of Dok to phenylalanine completely abolished the association of Dok with NCK and partially disrupted that with rasGAP. During the course of our study, activated Eph receptors were shown to induce tyrosine phosphorylation of Dok and its subsequent association with NCK (Holland *et al.*, 1997). Thus, the function of Dok may be to recruit rasGAP or NCK to a site near the plasma membrane in response to cell adhesion or insulin. The rasGAP protein has been thought to function as a negative regulator of Ras (Boguski and McCormick, 1993). The overexpression of wild-type Dok markedly enhanced the insulin-induced recruitment of rasGAP to cellular membranes, where Ras might be localized. Nevertheless, such overexpression did not affect the activation of MAP kinase in response to insulin. Therefore, it is unlikely that rasGAP complexed with tyrosine-phosphorylated Dok in the cell membrane functions to downregulate Ras in the insulin signaling pathway.

Overexpression of wild-type Dok markedly enhanced cell migration on a fibronectin-coated membrane even under unstimulated conditions. Moreover, it further increased insulin-stimulated cell migration on fibronectin. In contrast, overexpression of Dok Δ PH did not affect this response. It has been shown previously that Tyr960 of the insulin receptor is indispensable for insulin stimulation of cell migration (Yenush *et al.*, 1994). Together, our results indicate that tyrosine phosphorylation of Dok may play a critical role in linking insulin receptor activation to stimulation of cell migration on ECM components such as fibronectin. This notion was further supported by the fact that overexpression of a kinase-inactive mutant of Csk, which enhanced tyrosine phosphorylation of Dok, markedly increased cell migration on fibronectin under either basal or insulin-stimulated conditions. In addition, PP1, which decreased the tyrosine phosphorylation of Dok, markedly decreased these responses. We have observed that insulin stimulation did not substantially increase tyrosine phosphorylation of Dok in CHO-Csk-K/R cells (data not shown), since it may already be maximal under unstimulated condition. However, insulin stimulation further increased the migration rate of CHO-Csk-K/R cells (Figure 8B). It is likely that there might be another signaling pathway(s) that leads to cell migration in response to insulin but does not involve Dok. In insulin-stimulated cells, such a signaling pathway may function independently of, or act in concert with, the pathway that involves Dok, optimally inducing cell migration. Overexpression of DokY361F did not affect insulin-induced cell migration either. Therefore, it is possible that the Dok–NCK or Dok–rasGAP complex may mediate enhancement of insulin-induced cell migration in cells overexpressing wild-type Dok, although we cannot exclude the possibility that mutation of Tyr361 abolishes the association of Dok with other unidentified molecules.

NCK interacts, through its SH3 domains, with the serine-threonine kinase Pak (Bokoch *et al.*, 1996; Galisteo *et al.*, 1996), PRK2 (Quilliam *et al.*, 1996) and the multidomain protein WASP (Rivero-Lezcano *et al.*, 1995; Symons *et al.*, 1996), all of which could potentially contribute to reorganization of the actin cytoskeleton. Moreover, Dock, a *Drosophila* homolog of NCK, plays an important role in the signaling pathway that regulates growth cone motility (Garrity *et al.*, 1996). In addition, rasGAP has been implicated in regulation of the actin cytoskeleton (McGlade *et al.*, 1993). Thus, tyrosine-phosphorylated Dok may recruit downstream molecules that interact with NCK, such as Pak, PRK2 or WASP, to the actin cytoskeleton in response to cell adhesion or insulin. These downstream molecules might induce polymerization or depolymerization of actin filaments, and consequent reorganization of the actin cytoskeleton, eventually resulting in enhancement of cell migration.

Although insulin has previously been shown to stimulate cell motility (Yenush *et al.*, 1994), the molecular mechanism underlying this effect has remained largely unknown. Whereas phosphorylation of Tyr960 of the insulin receptor is required for this response, the overexpression of IRS-1 did not enhance insulin-stimulated cell migration (Yenush *et al.*, 1994). We have now shown that tyrosine phosphorylation of Dok may contribute to insulin-induced cell migration. Insulin is thought to achieve its multiple biological

effects by inducing the tyrosine phosphorylation of insulin receptor substrates such as IRS-1 to -4, Gab1 and Dok (White, 1998). All these proteins share common structures that are characterized by multiple tyrosine phosphorylation sites for the binding of SH2 domain-containing signaling molecules, a PH domain and a PTB domain (White, 1998). Grb2, SHP-2 and the 85-kDa subunit of PI 3-kinase preferentially bind, through their SH2 domains, to tyrosine-phosphorylated IRS-1, IRS-2 or Gab1 in response to insulin (White, 1998). Either IRS-1–Grb2 or IRS-1–SHP-2 complexes mediate insulin activation of the Ras–Raf–MEK–MAP kinase cascade that leads to cell proliferation (Skolnik *et al.*, 1993a,b; Noguchi *et al.*, 1994; Ogawa *et al.*, 1998). The complex of the 85-kDa subunit of PI 3-kinase with IRS-1 is thought to mediate insulin stimulation of glucose transport and membrane ruffling (Hara *et al.*, 1994; Kotani *et al.*, 1994). However, these SH2 domain-containing proteins did not bind to tyrosine-phosphorylated Dok in response to insulin. In contrast, rasGAP and NCK interacted preferentially with Dok in response to insulin; they showed little interaction with IRS-1 (T.Noguchi, T.Matozaki and M.Kasuga, unpublished data). Thus, insulin induces the tyrosine phosphorylation of various docking proteins, each of which then binds distinct SH2 domain-containing molecules, eliciting a variety of biological responses.

Materials and methods

Cells and antibodies

CHO-K1 cells and CHO cell lines overexpressing wild-type human insulin receptors (CHO-IR cells) or mutant receptors in which Tyr960 was replaced by alanine (CHO-IRY960A cells) were maintained in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS). CHO-IR cells that overexpress wild-type Csk (CHO-Csk-WT cells) and a kinase-negative Csk (CHO-Csk-K/R cells) were generated previously (Tsuda *et al.*, 1998).

CHO-IR cells that overexpress mutant Dok proteins were generated as follows. We introduced a point mutation that changed Tyr361 to phenylalanine into the mouse Dok cDNA by site-directed mutagenesis. The full-length wild-type and mutant Dok cDNAs were then inserted separately into the *Hind*III site of the pRc/CMV expression vector. A mutant Dok cDNA in which the nucleotide sequence encoding the 80 amino acids from Phe17 to Arg96 was deleted was generated by removing the fragment released by digestion of the full-length wild-type cDNA with *Eco*47III. All expression plasmids had been modified by incorporation of the nucleotide sequence encoding the HA epitope between the first and second codons of the open reading frame of the Dok cDNA (Yamanashi and Baltimore, 1997). CHO-IR cells ($\sim 2 \times 10^5$ cells per 6-cm dish) were transfected with both 5 μ g of pRc/CMV containing Dok cDNA and 1 μ g of pHyg, which contains the hygromycin B phosphotransferase gene, with the use of a CellPfect Transfection Kit (Amersham Pharmacia Biotech.). The cells were cultured in Ham's F12 medium supplemented with hygromycin B (300 μ g/ml) (Wako, Osaka, Japan) and 10% FBS, and colonies were isolated 14 days after transfection. Several cell lines overexpressing wild-type or mutant Dok proteins were identified by immunoblot analysis of cell lysates with polyclonal antibodies to Dok as described below.

Src family kinase-deficient (Src^{-/-} or Fyn^{-/-}) cells were isolated from the corresponding 'knockout' mouse embryos and immortalized with simian virus 40 large T antigen as described previously (Thomas *et al.*, 1995); they were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. 3Y1 rat fibroblasts transformed by v-Src (SR-3Y1 cells) were obtained from the Japanese Cancer Research Resources Bank and were also cultured in DMEM supplemented with 10% FBS.

To generate polyclonal antibodies to Dok, we injected female rabbits with a glutathione S-transferase (GST) fusion protein containing the C-terminal region of Dok. The mAb 2C4 to Dok was generated as described previously (Hosomi *et al.*, 1994). Rabbit polyclonal antibodies

to SHP-2 were generated against a GST fusion protein containing the C-terminal region of SHP-2 as described previously (Noguchi *et al.*, 1994). Rabbit polyclonal antibodies ($\alpha 91$) to both p44 and p42 MAP kinases were prepared against a synthetic peptide corresponding to residues 307 to 327 of rat MAP kinase. HRP-conjugated mAb PY20 to phosphotyrosine, a mAb to rasGAP (B4F8), and polyclonal antibodies to Fak, Grb2, Csk and c-Abl were obtained from Santa Cruz Biotechnology. The 12CA5 mAb to the HA epitope tag was purified from the culture supernatant of 12CA5 hybridoma cells. The mAb to NCK used for immunoblot analysis was from Transduction Laboratories; mAb 6C3 to NCK used for immunoprecipitation was generated in our laboratory with a GST fusion protein containing full-length NCK as antigen.

Cell adhesion and insulin stimulation

In adhesion experiments, cells were incubated for 12 h with culture medium in the absence of serum. Adhesion of CHO-IR or SR-3Y1 cells to the culture dish was partially disrupted by incubation in PBS in the absence of Ca^{2+} for 5–15 min at 37°C. CHO-IR, Src^{-/-} or Fyn^{-/-} cells, or the corresponding wild-type cells, were also detached from the dish by treatment with 0.025% trypsin, collected by centrifugation, and washed once with serum-free medium. The cells were replated on polystyrene dishes ($\sim 2.5 \times 10^7$ cells per 10-cm dish) coated with fibronectin (Sigma), laminin (Falcon), poly-L-lysine (Sigma) or BSA, and were then incubated for 1 h at 37°C in serum-free Ham's F12 medium or DMEM.

CHO-IR cells or CHO-IR cells that stably or transiently overexpressed Dok proteins were deprived of serum for 12 h, treated with or without 10 μM PP1 (Calbiochem) for 30 min, and then incubated for 5 min in the absence or presence of 100 nM insulin. Serum-deprived CHO-IR cells were also either detached from the culture dish or not before stimulation with insulin.

Immunoprecipitation and immunoblot analysis

After aspiration of culture medium, cells were immediately washed with ice-cold PBS and frozen in liquid nitrogen. The cells (in one 10-cm dish) were subsequently thawed on ice in 1 ml of ice-cold lysis buffer (20 mM Tris-HCl pH 7.6, 140 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) containing 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (10 $\mu\text{g}/\text{ml}$) and 1 mM sodium vanadate. The cell lysates were centrifuged at 10 000 g for 15 min at 4°C, and the resulting supernatants were subjected to immunoprecipitation and immunoblot analysis. For immunoprecipitation, the supernatants were incubated for 3 h at 4°C with various antibodies bound to protein G-Sepharose beads (20 μl of beads) (Amersham Pharmacia Biotech.). The beads were then washed three times with 1 ml of WG buffer (50 mM HEPES-NaOH pH 7.6, 150 mM NaCl, 0.1% Triton X-100), suspended in SDS sample buffer and boiled for 5 min. Gel electrophoresis and immunoblot analysis with various antibodies and the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech.) were performed as described previously (Noguchi *et al.*, 1994).

Subcellular fractionation

The following procedures were all performed at 4°C. Cells (in four 10-cm dishes) were treated with or without insulin were scraped into 2 ml of an ice-cold solution containing 20 mM HEPES-NaOH (pH 7.6), 5 mM sodium pyrophosphate, 5 mM EGTA, 250 mM sucrose, 5 mM NaF, 1 mM PMSF, aprotinin (10 $\mu\text{g}/\text{ml}$), and 1 mM sodium vanadate. The cells were homogenized with a Dounce homogenizer, and the homogenate was centrifuged at 900 g for 10 min. The resulting pellet was saved as the nuclear fraction, and the supernatant was centrifuged again at 100 000 g for 60 min. The second supernatant was saved as the cytosolic fraction, whereas the pellet was lysed in 1 ml of membrane solubilization buffer (20 mM Tris-HCl pH 7.5, 1% Triton X-100, 100 mM NaCl, 1 mM MgCl_2 , 1 mM CaCl_2) containing 5 mM NaF, 1 mM PMSF, aprotinin (10 $\mu\text{g}/\text{ml}$), and 1 mM sodium vanadate. The resulting extract was centrifuged at 10 000 g for 10 min, yielding a pellet referred to as the cytoskeletal fraction and a supernatant that was centrifuged again at 100 000 g for 60 min. The final supernatant and pellet were saved as the membrane fraction and the membrane-skeleton fraction, respectively.

Expression and purification of recombinant Dok

A recombinant C-terminal fragment of Dok was generated with the GST fusion protein-expression system. The polymerase chain reaction (PCR) was performed with wild-type human Dok cDNA as a template as well as 5'-TAGGATCCCGGGAGCCCAAGGATGCATGGTGG (nucleotides 1163–1186) and 5'-TCGAATTCTCAGGTAGAGCCCTCTGACTTGAC

(nucleotides 1446–1468) as sense and antisense primers, respectively. The amplification products were digested with *Bam*HI and *Eco*RI, and inserted in frame into the *Bam*HI and *Eco*RI sites of the vector pGEX-2T (Amersham Pharmacia Biotech.). The encoded GST fusion protein was expressed and purified with glutathione-Sepharose beads (Amersham Pharmacia Biotech.) as described previously (Noguchi *et al.*, 1994).

Cell migration assay

Migration of CHO-IR cells was assessed with a modified Boyden chamber assay essentially as described previously (Horikawa *et al.*, 1995). Polyvinylpyrrolidone-free filters (pore size, 8 μm ; Neuroprobe) were coated for 30 min with fibronectin (13.3 $\mu\text{g}/\text{ml}$) diluted in serum-free Ham's F12 medium. A coated filter was dried and placed over the lower well of a Boyden chamber that had been filled with serum-free Ham's F12 medium supplemented or not with 5 nM insulin. The gasket and upper part of the chamber were then assembled. After detachment from the culture dish by exposure to trypsin and subsequent dilution, 1.5×10^5 cells in 0.2 ml of serum-free medium were added to the upper well. In some experiments PP1 was added to both upper and lower parts of the chamber at a final concentration of 10 μM . The chamber was then placed in a CO_2 incubator, and the cells were incubated for 3 h at 37°C. Cells that had migrated were fixed in methanol, washed with PBS and exposed to Giemsa stain for 15 s (Nakarai Tesque). After washing in water, the filter was mounted on a glass slide. The side of the filter to which the cells had been added was scraped. All cells within the area of the lower side of the membrane representing the well were counted visually under a microscope fitted with a grid eyepiece, at a total magnification of $\times 200$.

Assay of MAP kinase activation

MAP kinase activation was monitored by immunoblot analysis of cell lysates with antibodies to p44 and p42 isoforms of MAP kinase (New England Biolabs) that recognize the enzyme only when it is activated by phosphorylation of Tyr204.

Acknowledgements

We thank B.Stillman for providing the human Dok cDNA, A.Imamoto and P.Soriano for Src^{-/-} and Fyn^{-/-} cells, and T.Horikawa for help with the cell migration assay. This work was supported by a grant-in-aid for cancer research and a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan, a grant-in-aid for the Research for the Future Program from the Japan Society for the Promotion of Science, and grants from the Princess Takamatsu Cancer Research Fund, the Yamanouchi Foundation for Research on Metabolic Disorders and the Takeda Bioscience Foundation.

References

- Bockholt,S.M. and Burridge,K. (1993) Cell spreading on extracellular matrix proteins induces tyrosine phosphorylation of tensin. *J. Biol. Chem.*, **268**, 14565–14567.
- Boguski,M.S. and McCormick,F. (1993) Proteins regulating Ras and its relatives. *Nature*, **366**, 643–654.
- Bokoch,G.M., Wang,Y., Bohl,B.P., Sells,M.A., Quilliam,L.A. and Knaus,U.G. (1996) Interaction of the Nck adapter protein with p21-activated kinase (PAK1). *J. Biol. Chem.*, **271**, 25746–25749.
- Bouton,A.H., Kanner,S.B., Vines,R.R., Wang,H.C., Gibbs,J.B. and Parsons,J.T. (1991) Transformation by pp60src or stimulation of cells with epidermal growth factor induces the stable association of tyrosine-phosphorylated cellular proteins with GTPase-activating protein. *Mol. Cell. Biol.*, **11**, 945–953.
- Burridge,K., Turner,C.E. and Romer,L.H. (1992) Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J. Cell Biol.*, **119**, 893–903.
- Carpino,N., Wisniewski,D., Strife,A., Marshak,D., Kobayashi,R., Stillman,B. and Clarkson,B. (1997) p62^{dok}: a constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells. *Cell*, **88**, 197–204.
- Cobb,B.S., Schaller,M.D., Leu,T.-H. and Parsons,J.T. (1994) Stable association of pp60src and pp59fyn with the focal adhesion-associated protein tyrosine kinase, pp125FAK. *Mol. Cell. Biol.*, **14**, 147–155.

- Cristofano, A.D., Carpino, N., Dunant, N., Friedland, G., Kobayashi, R., Strife, A., Wisniewski, D., Clarkson, B., Pandolfi, P.P. and Resh, M.D. (1998) Molecular cloning and characterization of p56^{dok-2} defines a new family of RasGAP-binding proteins. *J. Biol. Chem.*, **273**, 4827–4830.
- Danielsen, A.G. and Roth, R.A. (1996) Role of the juxtamembrane tyrosine in insulin receptor-mediated tyrosine phosphorylation of p60 endogenous substrates. *Endocrinology*, **137**, 5326–5331.
- Daub, H., Wallasch, C., Lankenau, A., Herrlich, A. and Ullrich, A. (1997) Signal characteristics of G protein-transactivated EGF receptor. *EMBO J.*, **16**, 7032–7044.
- DeClue, J.E., Vass, W.C., Johnson, M.R., Stacey, D.W. and Lowy, D.R. (1993) Functional role of GTPase-activating protein in cell transformation by pp60v-src. *Mol. Cell. Biol.*, **13**, 6799–6809.
- de Vries-Smits, A.M.M., Burgering, B.M.T., Leenders, S.J., Marshall, C.J. and Bos, J.L. (1992) Involvement of p21ras in activation of extracellular signal-regulated kinase 2. *Nature*, **357**, 602–604.
- Eck, M.J., Dhe-Paganon, S., Trub, T., Nolte, R.T. and Shoelson, S.E. (1996) Structure of the IRS-1 PTB domain bound to the juxtamembrane region of the insulin receptor. *Cell*, **85**, 695–705.
- Ellis, C., Moran, M., McCormick, F. and Pawson, T. (1990) Phosphorylation of GAP and GAP-associated proteins by transforming and mitogenic tyrosine kinases. *Nature*, **343**, 377–381.
- Fujioka, Y., Matozaki, T., Noguchi, T., Iwamatsu, A., Yamao, T., Takahashi, N., Tsuda, M., Takada, T. and Kasuga, M. (1996) A novel membrane glycoprotein, SHPS-1, that binds the SH2-domain-containing protein tyrosine phosphatase SHP-2 in response to mitogens and cell adhesion. *Mol. Cell. Biol.*, **16**, 6887–6899.
- Galisteo, M.L., Chernoff, J., Su, Y.-C., Skolnik, E.Y. and Schlessinger, J. (1996) The adaptor protein Nck links receptor tyrosine kinase with the serine-threonine kinase Pak1. *J. Biol. Chem.*, **271**, 20997–21000.
- Garrity, P.A., Rao, Y., Salecker, I., McGlade, J., Pawson, T. and Zipursky, S.L. (1996) *Drosophila* photoreceptor axon guidance and targeting requires the deadlocks SH2/SH3 adapter protein. *Cell*, **85**, 639–650.
- Hanks, S.K. and Polte, T.R. (1997) Signaling through focal adhesion kinase. *BioEssays*, **19**, 137–145.
- Hara, K. et al. (1994) 1-Phosphatidylinositol 3-kinase activity is required for insulin-stimulated glucose transport but not for RAS activation in CHO cells. *Proc. Natl Acad. Sci. USA*, **91**, 7415–7419.
- He, W., O'Neil, T.J. and Gustafson, T.A. (1995) Distinct modes of interaction of SHC and insulin receptor substrate-1 with the insulin receptor NPEY region via non-SH2 domains. *J. Biol. Chem.*, **270**, 23258–23262.
- Holgado-Madruga, M., Emlet, D.R., Moscatello, D.K., Godwin, A.K. and Wong, A.J. (1996) A Grb2-associated docking protein in EGF- and insulin-receptor signalling. *Nature*, **379**, 560–564.
- Holland, S.J., Gale, N.W., Gish, G.D., Roth, R.A., Songyang, Z., Cantley, L.C., Henkemeyer, M., Yancopoulos, G.D. and Pawson, T. (1997) Juxtamembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells. *EMBO J.*, **16**, 3877–3888.
- Horikawa, T., Norris, D.A., Yohn, J.J., Zekman, T., Travers, J.B. and Morelli, J.G. (1995) Melanocyte mitogens induce both melanocyte chemokinesis and chemotaxis. *J. Invest. Dermatol.*, **104**, 256–259.
- Hosomi, Y. et al. (1994) Characterization of a 60-kilodalton substrate of the insulin receptor kinase. *J. Biol. Chem.*, **269**, 11498–11502.
- Kotani, K. et al. (1994) Involvement of phosphoinositide 3-kinase in insulin- or IGF-1-induced membrane ruffling. *EMBO J.*, **13**, 2313–2321.
- Lavan, B.E., Fantin, V.R., Chang, E.T., Lane, W.S., Keller, S.R. and Lienhard, G.E. (1997a) A novel 160-kDa phosphotyrosine protein in insulin-treated embryonic kidney cells is a new member of the insulin receptor substrate family. *J. Biol. Chem.*, **272**, 21403–21407.
- Lavan, B.E., Lane, W.S. and Lienhard, G.E. (1997b) The 60 kDa phosphotyrosine protein in insulin-treated adipocytes is a new member of the insulin receptor substrate family. *J. Biol. Chem.*, **272**, 11439–11443.
- Lemmon, M.A., Ferguson, K.M. and Schlessinger, J. (1996) PH domains: diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell*, **85**, 621–624.
- Lugo, T.G., Pendergast, A.M., Muller, A.J. and Witte, O.N. (1990) Tyrosine kinase activity and transformation potency of *bcr-abl* oncogene products. *Science*, **247**, 1079–1082.
- McGlade, J., Brunkhorst, B., Anderson, D., Mbamalu, G., Settleman, J., Dedhar, S., Rozakis-Adcock, M., Chen, L.B. and Pawson, T. (1993) The N-terminal region of GAP regulates cytoskeletal structure and cell adhesion. *EMBO J.*, **12**, 3073–3081.
- Mitchison, T.J. and Cramer, L.P. (1996) Actin-based cell motility and cell locomotion. *Cell*, **84**, 371–379.
- Moran, M.F., Polakis, P., McCormick, F., Pawson, T. and Ellis, C. (1991) Protein tyrosine kinases regulate the phosphorylation, protein interactions, subcellular distribution and activity of p21ras GTPase-activating protein. *Mol. Cell. Biol.*, **11**, 1804–1812.
- Musacchio, A., Gibson, T., Rice, P., Thompson, J. and Saraste, M. (1993) The PH domain: a common piece in the structural patchwork of signaling proteins. *Trends Biochem. Sci.*, **18**, 343–348.
- Myers, M.G., Jr., Grammer, T.C., Brooks, J., Glasheen, E.M., Wang, L.M., Sun, X.J., Blenis, J., Pierce, J.H. and White, M.F. (1995) The pleckstrin homology domain in insulin receptor substrate-1 sensitizes insulin signaling. *J. Biol. Chem.*, **270**, 11715–11718.
- Nada, S., Okada, M., MacAuley, A., Cooper, J.A. and Nakagawa, H. (1991) Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60c-src. *Nature*, **351**, 69–72.
- Neet, K. and Hunter, T. (1995) The nonreceptor protein-tyrosine kinase csk complexes directly with the GTPase-activating protein-associated p62 in cells expressing v-src or activated c-src. *Mol. Cell. Biol.*, **15**, 4908–4920.
- Nelms, K., Snow, A.L., Hu-Li, J. and Paul, W.E. (1998) FRIP, a hematopoietic cell-specific rasGAP-interacting protein phosphorylated in response to cytokine stimulation. *Immunity*, **9**, 13–24.
- Noguchi, T., Matozaki, T., Horita, K., Fujioka, Y. and Kasuga, M. (1994) Role of SH-PTP2, a protein-tyrosine phosphatase with Src homology 2 domains, in insulin-stimulated Ras activation. *Mol. Cell. Biol.*, **14**, 6674–6682.
- Nojima, Y. et al. (1995) Integrin-mediated cell adhesion promotes tyrosine phosphorylation of p130Cas, a Src homology 3-containing molecule having multiple Src homology 2-binding motifs. *J. Biol. Chem.*, **270**, 15398–15402.
- Ogawa, W., Hosomi, Y., Shii, K. and Roth, R.A. (1994) Evidence for two distinct 60-kilodalton substrates of the SRC tyrosine kinase. *J. Biol. Chem.*, **269**, 29602–29608.
- Ogawa, W., Matozaki, T. and Kasuga, M. (1998) Role of binding proteins to IRS-1 in insulin signalling. *Mol. Cell. Biochem.*, **182**, 13–22.
- Okada, M., Nada, S., Yamanashi, Y., Yamamoto, T. and Nakagawa, H. (1991) CSK: a protein-tyrosine kinase involved in regulation of src family kinases. *J. Biol. Chem.*, **266**, 24249–24252.
- Quilliam, L.A. et al. (1996) Isolation of a NCK-associated kinase, PRK2, an SH3-binding protein and potential effector of Rho protein signaling. *J. Biol. Chem.*, **271**, 28772–28776.
- Rivero-Lezcano, O.M., Marcilla, A., Sameshima, J.H. and Robbins, K.C. (1995) Wiskott-Aldrich syndrome protein physically associates with NCK through Src homology 3 domains. *Mol. Cell. Biol.*, **15**, 5725–5731.
- Sanchez-Margalet, V., Zoratti, R. and Sung, C.K. (1995) Insulin-like growth factor-1 stimulation of cells induces formation of complexes containing phosphatidylinositol-3-kinase, guanosine triphosphatase-activating protein (GAP) and p62 GAP-associated protein. *Endocrinology*, **136**, 316–321.
- Schlaepfer, D.D., Hanks, S.K., Hunter, T. and van der Geer, P. (1994) Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature*, **372**, 786–791.
- Skolnik, E.Y., Batzer, A., Li, N., Lee, C.-H., Lowenstein, E., Mohammadi, M., Morgolis, B. and Schlessinger, J. (1993a) The function of GRB2 in linking the insulin receptor to Ras signaling pathways. *Science*, **260**, 1953–1955.
- Skolnik, E.Y. et al. (1993b) The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS1 and Shc: implications for insulin control of Ras signaling. *EMBO J.*, **12**, 1929–1936.
- Songyang, Z. et al. (1993) SH2 domains recognize specific phosphopeptide sequences. *Cell*, **72**, 767–778.
- Songyang, Z. et al. (1995) Catalytic specificity of protein tyrosine kinases is critical for selective signalling. *Nature*, **373**, 536–539.
- Sun, X.J., Rothenberg, P., Kahn, C.R., Backer, J.M., Araki, E., Wilden, P.A., Cahill, D.A., Goldstein, B.J. and White, M.F. (1991) The structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature*, **352**, 73–77.
- Sun, X.J., Wang, L.-H., Zang, Y., Yenush, L., Myers, M.G., Jr., Glasheen, E., Lane, W.S., Pierce, J.H. and White, M.F. (1995) Role of IRS-2 in insulin and cytokine signalling. *Nature*, **377**, 173–177.

- Symons,M., Derry,J.M.J., Karlak,B., Jiang,S., Lemahieu,V., McCormick,F., Francke,U. and Abo,A. (1996) Wiskott–Aldrich syndrome protein, a novel effector for the GTPase CDC42Hs, is implicated in actin polymerization. *Cell*, **84**, 723–734.
- Thomas,S.M., Soriano,P. and Imamoto,A. (1995) Specific and redundant roles of Src and Fyn in organizing the cytoskeleton. *Nature*, **376**, 267–271.
- Tsuda,M. *et al.* (1998) Integrin-mediated tyrosine phosphorylation of SHPS-1 and its association with SHP-2. *J. Biol. Chem.*, **273**, 13223–13229.
- Voliyovitch,H., Schindler,D.G., Hadari,Y.R., Taylor,S.I., Accili,D. and Zick,Y. (1995) Tyrosine phosphorylation of insulin receptor substrate-1 *in vivo* depends upon the presence of its pleckstrin homology region. *J. Biol. Chem.*, **270**, 18083–18087.
- Vuica,B.M., Desiderio,S. and Schneck,J.P. (1997) Differential effects of B cell receptor and B cell receptor-FcγRIIB1 engagement on docking of Csk to GTPase-activating protein (GAP)-associated p62. *J. Exp. Med.*, **186**, 259–267.
- White,M.F. (1998) The IRS-signalling system: a network of docking proteins that mediate insulin action. *Mol. Cell. Biochem.*, **182**, 3–11.
- White,M.F., Livingston,J.N., Backer,J.M., Lauris,V., Dull,T.J., Ullrich,A. and Kahn,C.R. (1988) Mutation of the insulin receptor at tyrosine 960 inhibits signal transmission but does not affect its tyrosine kinase activity. *Cell*, **54**, 641–649.
- Wisniewski,D., Strife,A., Berman,E. and Clarkson,B. (1996) c-kit ligand stimulates tyrosine phosphorylation of a similar pattern of phosphotyrosyl proteins in primary primitive normal hematopoietic progenitors that are constitutively phosphorylated in comparable primitive progenitors in chronic phase chronic myelogenous leukemia. *Leukemia*, **10**, 229–237.
- Wolf,G., Trub,T., Ottinger,E., Groninga,L., Lynch,A., White,M.F., Miyazaki,M., Lee,J. and Shoelson,S.E. (1995) PTB domains of IRS-1 and Shc have distinct but overlapping binding specificities. *J. Biol. Chem.*, **270**, 27407–27410.
- Yamada,K.M. and Miyamoto,S. (1995) Integrin transmembrane signaling and cytoskeletal control. *Curr. Opin. Cell Biol.*, **7**, 681–689.
- Yamanashi,Y. and Baltimore,D. (1997) Identification of the Abl- and rasGAP-associated 62 kDa protein as a docking protein, Dok. *Cell*, **88**, 205–211.
- Yenush,L., Kundra,V., White,M.F. and Zetter,B.R. (1994) Functional domains of the insulin receptor responsible for chemotactic signaling. *J. Biol. Chem.*, **269**, 100–104.
- Yonezawa,K. *et al.* (1994) Signal transduction pathways from insulin receptors to Ras. Analysis by mutant insulin receptors. *J. Biol. Chem.*, **269**, 4634–4640.
- Zhou,M.-M. *et al.* (1995) Structure and ligand recognition of the phosphotyrosine binding domain of Shc. *Nature*, **378**, 584–592.

Received October 21, 1998; revised and accepted February 18, 1999