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Tyrosine phosphorylation of ACK in response to temperature shift-down, hyperosmotic shock, and epidermal growth factor stimulation

Takaya Satoh*, Juran Kato, Kazuhiko Nishida, Yoshito Kaziro

Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226, Japan

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Abstract The mammalian Cdc42 protein regulates various kinds of cellular responses, including formation of filopodia, polarization of T cells, and cell cycle progression. A non-receptor tyrosine kinase ACK, which specifically binds to the GTP-bound form of Cdc42, was isolated as a putative target of Cdc42. Here we show the induction of tyrosine phosphorylation of ACK in response to temperature shift-down to 25°C, and hypertonic shock, as well as stimulation with epidermal growth factor (EGF) in human embryonic kidney (HEK) 293 cells. The increased tyrosine phosphorylation level upon temperature shift-down was sustained for at least 60 min, whereas reversion of the temperature to 37°C caused rapid tyrosine dephosphorylation to the initial level. The responses to EGF and the high osmolarity were transient. Furthermore, we observed association of ACK with an adaptor protein Grb2, which may suggest the involvement of Grb2 in EGF receptor-mediated tyrosine phosphorylation of ACK.

Key words: ACK; Cdc42; Tyrosine phosphorylation; Temperature shift-down; Hyperosmolarity

1. Introduction

The mammalian Cdc42 protein belongs to the Rho family of low molecular weight GTP-binding proteins [1,2]. Recent works employing the technique of microinjection have demonstrated that an active Cdc42 protein induced the formation of peripheral actin microspikes termed filopodia [3], while a dominant-negative Cdc42 interfered with bradykinin-promoted formation of filopodia [4]. Moreover, an activated Cdc42 led to the formation of lamellipodia and actin stress fibers, which are known to be regulated by other members of Rho family GTPases, Rac and Rho, respectively [3]. From these results, a signal transduction cascade consisting of Cdc42, Rac, and Rho has been proposed [3,5]. Additionally, in T cell activation signaling, Cdc42 regulates cell polarization towards antigen-presenting cells [6]. Cdc42 as well as Rho and Rac proteins are critical components not only in the regulation of cytoskeletal organization, but also in the signal transduction for DNA synthesis [7].

Recent progress in the search for targets of small GTP-binding proteins has revealed that one GTP-binding protein directly interacts with a variety of proteins, thereby regulating various kinds of signaling pathways. For Cdc42, a number of molecules including Ste20/PAK serine/threonine kinases [8-11], ACK tyrosine kinase [12], phosphatidylinositol-3-kinase [13,14], and $p67^{phox}$ of NADPH oxidase [15] have been identified as direct targets. Moreover, putative target proteins have been proposed based on their structural feature identified as a Cdc42/Rac-binding motif [16]. Among them, Ste20/PAK kinases have recently been characterized well. In *Saccharomyces cerevisiae*, the signal from mating pheromones is transmitted through Cdc42 and the mitogen-activated protein kinase (MAPK) cascade involving Ste20, Ste11, Ste7, and Fus3/Kss1 kinases [17]. Likewise, in mammalian cells, Cdc42 activates PAKs, and then, PAKs stimulate a signaling pathway leading to the activation of stress-activated protein kinases (SAPKs)/c-Jun amino-terminal kinases (JNKs) ([7,18-20], see also [21] for a review.)

In contrast with the Ste20/PAK pathway, the role of ACK-mediated signal transduction is obscure. ACK was first isolated as a protein that bound Cdc42 but not Rac or Rho, whereas PAKs, phosphatidylinositol-3-kinase, and $p67^{phox}$ interact with Rac as well as Cdc42. The binding of ACK to Cdc42 is GTP-dependent, suggesting that ACK may be a target of Cdc42 although the modulation of the tyrosine kinase activity of ACK upon binding to Cdc42·GTP has not been clarified [12]. Moreover, attenuation of the intrinsic as well as Bcr-promoted GTPase activities of Cdc42 by ACK has been demonstrated [12].

As a first step to reveal the function of ACK, we examined the tyrosine phosphorylation level of ACK in response to various extracellular stimuli in human embryonic kidney (HEK) 293 cells. During the screening of ligands that stimulate ACK tyrosine phosphorylation, it has been found that the addition of the control buffer alone to the cells at the room temperature caused a rapid and substantial increase of ACK tyrosine phosphorylation, and we ultimately identified the phenomenon as an effect of the temperature shift from 37°C to 25°C. Furthermore, high osmolarity and treatment with epidermal growth factor (EGF) were found to stimulate ACK tyrosine phosphorylation, suggesting that multiple signals may control the activity ACK. Also, the association of ACK with an adaptor protein Grb2 was shown, which implies the link between the EGF receptor and ACK through Grb2.

2. Materials and methods

2.1. Materials

An anti-ACK antibody (sc-323) and an epitope peptide of ACK (sc-323P) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against phosphotyrosine (05-321) and Shc (06-203) were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). An anti-Grb2 antibody (MS-20-3) and EGF (T001A) were purchased from MBL (Nagoya, Japan) and Takara Shuzo (Otsu, Japan), respectively. An anti-Src monoclonal antibody 327 was prepared from the concentrated culture supernatant of the hybridoma. A human full-length ACK cDNA clone [12] was a generous gift from Edward Manser and Louis Lim (Institute of Molecular Cell Biology, National

*Corresponding author. Fax: (81) (45) 924-5822.

Abbreviations: MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; JNK, c-Jun amino-terminal kinase; HEK, human embryonic kidney; EGF, epidermal growth factor

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University of Singapore, Singapore). An expression plasmid pCMV5-hACK was constructed by inserting the *Eco*RI-*Eco*RI fragment containing the whole encoding region of ACK into pCMV5 [22]. A human full-length Grb2 cDNA clone [23] was a generous gift from Andreas Batzer and Joseph Schlessinger (New York University Medical Center, NY). The *Hin*dIII-*Bam*HI fragment containing the Grb2 full-length cDNA was inserted into pCMV5 [22], and then the *Bst*XI-*Bst*XI fragment encoding the SH2 domain was removed, generating an expression plasmid termed pCMV5-hGrb2(ΔSH2).

2.2. Cell culture

HEK 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. For the analysis of responses to various extracellular stimuli, the cells were starved within a serum-free medium supplemented with 1 mg/ml bovine serum albumin for 24 h prior to stimulation.

2.3. DNA transfection

A plasmid DNA was introduced into HEK 293 cells according to a standard calcium phosphate-DNA coprecipitation method. Briefly, the precipitate of the calcium phosphate-DNA complex was generated by mixing a solution containing DNA and 250 mM calcium chloride with an equal volume of 2×HBS (50 mM HEPES-NaOH (pH 7.05), 280 mM NaCl, 1.5 mM Na_2HPO_4) under aspiration with air. The suspension was added to the cells cultured in a fresh medium. After 48 h incubation, the cells were harvested, and subjected to immunoprecipitation and immunoblotting.

2.4. Immunoprecipitation and immunoblotting

Cells were dissolved into IP buffer (50 mM HEPES-NaOH (pH 7.3), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 2 mM $MgCl_2$, 1 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin), and the supernatant resulting from centrifugation (15 000×*g*) for 5 min at 4°C was obtained. Proteins were immunoprecipitated from the lysate with specific antibodies and Protein A-Sepharose CL-4B (Phamacia Biotechnology, Inc.), and then analyzed by subsequent SDS-PAGE and immunoblotting using enhanced chemiluminescence detection reagents (DuPont NEN).

3. Results

3.1. Tyrosine phosphorylation of ACK in response to temperature shift-down

Fig. 1 shows an increase of the tyrosine phosphorylation level of ACK in HEK 293 cells following the temperature change of the culture medium from 37°C to 25°C. The culture medium was replaced by fresh medium preincubated at the temperature to be tested, and the culture plate was subjected to further incubation at the same temperature. Subsequently, tyrosine phosphorylation of the endogenous ACK was evaluated by immunoblotting with an anti-phosphotyrosine antibody following immunoprecipitation using an anti-ACK antibody within the lysates prepared from the control and stimulated cells. Fig. 1A demonstrates the effects of 10 min incubation at various temperatures. Marked enhancement of the tyrosine phosphorylation of ACK was observed at 25°C, whereas the temperature shift to 42°C or 0°C had no effect. The bands were detected dependent on the anti-ACK antibody for immunoprecipitation, and the same amounts of ACK were precipitated between stimulated and unstimulated cells (data not shown). Fig. 1B shows the time course of ACK tyrosine phosphorylation following the temperature decrease to 25°C. After 3 min incubation, a significant increase was detected, which persisted for at least 60 min. In contrast, the temperature increase to 42°C did not affect the level of tyrosine phosphorylation up to 60 min (data not shown). The tyrosine phosphorylation level of another non-receptor type

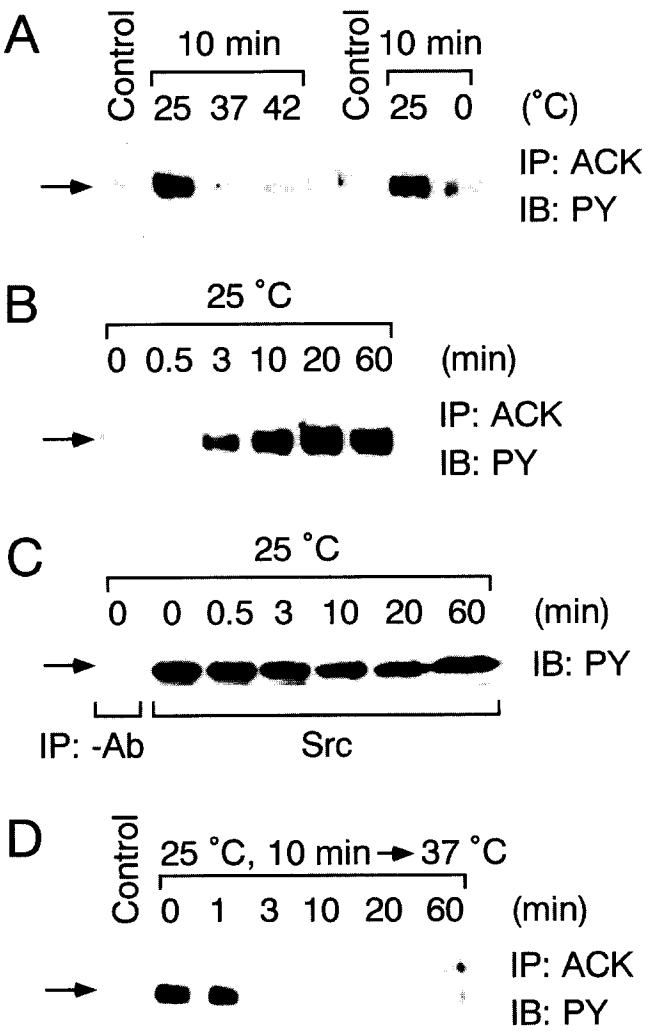

Fig. 1. Induction of ACK tyrosine phosphorylation in response to temperature shift from 37°C to 25°C. (A) Tyrosine phosphorylation of ACK following the shift to various temperatures. Lysates of HEK 293 cells (10^7 cells per lane) were prepared following no treatment (control) or 10 min incubation at various temperatures as indicated. Tyrosine phosphorylation of ACK was detected by immunoprecipitation with an anti-ACK antibody (sc-323, 0.4 μg) and subsequent immunoblotting with an anti-phosphotyrosine antibody (05-321, 2 μg/ml). (B) Time course of the increase of ACK tyrosine phosphorylation upon the temperature shift from 37°C to 25°C. Lysates were prepared from HEK 293 cells (10^7 cells per lane) incubated at 25°C for indicated periods, and tyrosine phosphorylation of ACK was detected as in (A). (C) Time course of tyrosine phosphorylation of c-Src upon the temperature shift from 37°C to 25°C. Lysates were prepared from the cells treated as in (B), and tyrosine phosphorylation of c-Src was detected by immunoprecipitation with an anti-c-Src antibody (327) and subsequent immunoblotting with an anti-phosphotyrosine antibody (05-321, 2 μg/ml). (D) Time course of the tyrosine dephosphorylation of ACK upon the temperature reversion from 25°C to 37°C. Lysates were prepared from HEK 293 cells (10^7 cells per lane) incubated at 37°C for indicated periods following preincubation at 25°C for 10 min. Tyrosine phosphorylation of ACK was detected as in (A).

tyrosine kinase c-Src during incubation at 25°C is demonstrated in Fig. 1C as a control, where no change in the level was detected. Therefore, it is likely that a specific regulatory mechanism for ACK tyrosine phosphorylation may exist. Fig. 1D shows the kinetics of tyrosine dephosphorylation of ACK after the temperature reversion to 37°C following incubation

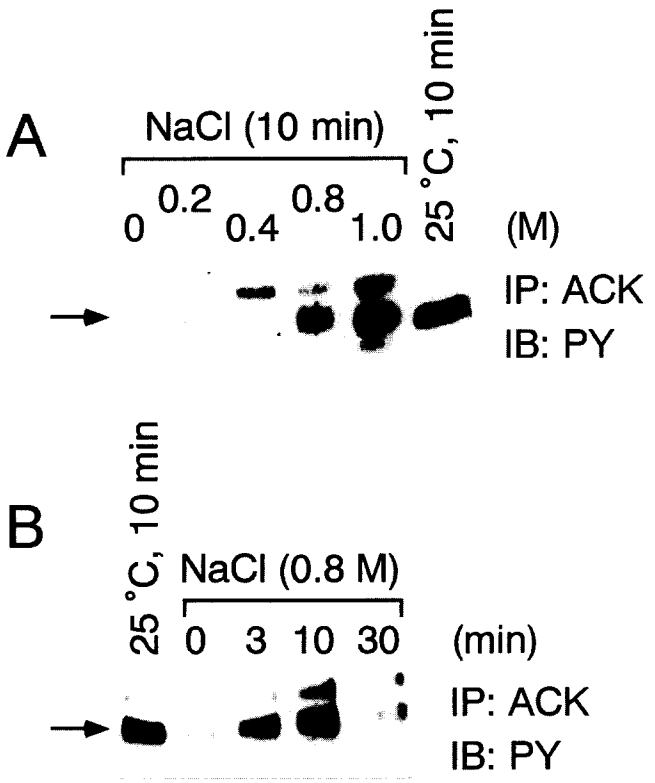

Fig. 2. Induction of ACK tyrosine phosphorylation in response to hyperosmolarity. (A) Tyrosine phosphorylation of ACK in the media containing various concentrations of NaCl. HEK 293 cells (10^7 cells per lane) were exposed to hypertonic media containing indicated concentrations of NaCl for 10 min at 37°C. Tyrosine phosphorylation of ACK was detected as in Fig. 1A. (B) Time course of the increase of ACK tyrosine phosphorylation upon hyperosmolarity. HEK 293 cells (10^7 cells per lane) were exposed to hypertonic medium containing 0.8 M NaCl at 37°C for the indicated periods. Tyrosine phosphorylation of ACK was detected as in Fig. 1A.

at 25°C for 10 min. The level of tyrosine phosphorylation rapidly decreased, and returned to the initial level after 3 min incubation.

3.2. *Tyrosine phosphorylation of ACK in response to hyperosmolarity*

The SAPK/JNK pathway, which is regulated by Cdc42 and Rac GTPases, is activated by various kinds of extracellular stress signals, including hyperosmolarity. Thus, we next examined the effects of hyperosmolarity on ACK tyrosine phosphorylation. As shown in Fig. 2A, hyperosmolarity was found to trigger enhanced tyrosine phosphorylation of ACK. The effect was observed after 10 min treatment at NaCl concentrations above 0.8 M which are similar to those that induce activation of SAPK/JNK or p38, a mammalian homologue of *S. cerevisiae* Hog1 [24,25]. However, the induction of ACK tyrosine phosphorylation by hyperosmolarity is transient, as shown in Fig. 2B in contrast to the case of SAPK/JNK, where the activation persists for more than 30 min. The upper bands seem unrelated to ACK, since their appearance is neither reproducible nor dependent on the anti-ACK antibody. Unlike ACK tyrosine kinase, the tyrosine phosphorylation level of endogenous c-Src was not affected by treatment with 0.8 M NaCl for 10 min (data not shown).

3.3. *Tyrosine phosphorylation of ACK in response to EGF stimulation*

Fig. 3 demonstrates an increase in the tyrosine phosphorylation level of ACK upon EGF treatment. EGF was added to the cells without replacing the culture medium to avoid any temperature change. Following 1 min treatment with EGF, enhancement of ACK tyrosine phosphorylation was detected, and thereafter, the tyrosine phosphorylation level rapidly fell. The maximum level of tyrosine phosphorylation was almost the same as that induced by the temperature change to 25°C.

3.4. *Complex formation between ACK and Grb2*

To gain further insight into the mechanism of EGF-stimulated ACK tyrosine phosphorylation, we tested the involvement of Grb2 and Shc, well-characterized adaptor proteins that link various receptor tyrosine kinases and their downstream signaling pathways, for instance, the mSos/Ras/Raf pathway [26,27]. Fig. 4A shows association of ACK with Grb2 in unstimulated HEK 293 cells. Immunoprecipitates of an anti-ACK antibody or a control rabbit IgG from unstimulated cells were subjected to immunoblotting with an anti-Grb2 antibody. Grb2 was detected within the anti-ACK immunoprecipitate, but not within the control. Moreover, addition of excess amounts of a peptide corresponding to the epitope for the anti-ACK antibody diminished the band of Grb2 almost completely. Then, we examined whether the interaction of ACK with Grb2 was affected by the addition of EGF. Larger amounts of Grb2 protein were coimmunoprecipitated with ACK on EGF stimulation, while the expression level and the amounts of ACK precipitated were almost identical between stimulated and unstimulated cells (Fig. 4B). Furthermore, we observed association of Shc with Grb2 in EGF-stimulated, but not in unstimulated cells (Fig. 4C) as previously reported in various cell lines. Collectively, it is possible that the stimulation of the cells with EGF may cause the formation of a signal transducing complex consisting of the EGF receptor, Shc, Grb2, and ACK.

Grb2 is a protein comprising a single SH2 domain flanked by two SH3 domains. Thus, it is important to know which of the domains is responsible for the ACK/Grb2 association. For this purpose, we made a deletion mutant of Grb2 protein, termed Grb2(ΔSH2), which lacks the whole SH2 domain and the amino-terminal 10 residues of the second SH3 domain (59P to 172D). An expression plasmid for Grb2(ΔSH2) (pCMV5-Grb2(ΔSH2)) was introduced into HEK 293 cells, and the lysate was prepared without stimulation. The expression level of Grb2(ΔSH2) was several-fold higher in comparison with the endogenous Grb2 as estimated by immunoblot-

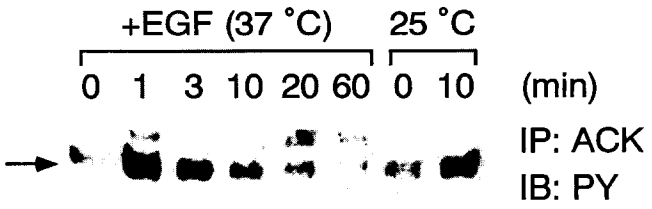

Fig. 3. Induction of ACK tyrosine phosphorylation in response to EGF stimulation. Time course of the increase of ACK tyrosine phosphorylation upon EGF stimulation is shown. Tyrosine phosphorylation of ACK in HEK 293 cells (10^7 cells per lane) stimulated with EGF (100 ng/ml) at 37°C for indicated periods was analyzed as in Fig. 1A.

ting with an antibody against the SH3 domain of Grb2 (data not shown). As shown in Fig. 4D, Grb2(ΔSH2) was able to be coimmunoprecipitated with ACK like the endogenous Grb2, indicating that the SH2 domain is dispensable for the ACK/Grb2 association, and either or both of the SH3 domains may be responsible for the interaction.

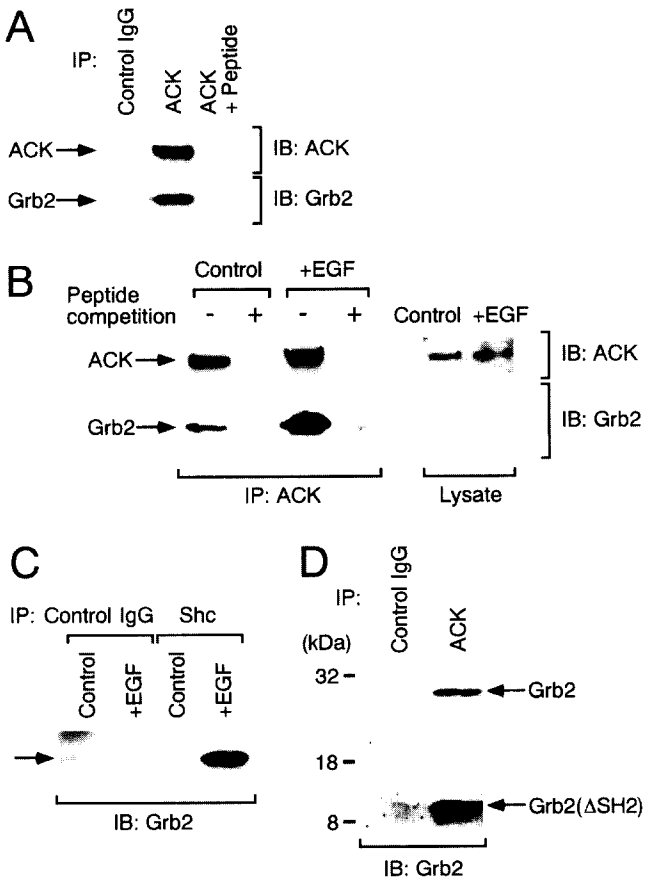

Fig. 4. Association of ACK with adaptor proteins. (A) Association of ACK with Grb2 in unstimulated HEK 293 cells. An expression plasmid for human ACK (pCMV5-hACK, 20 μg for 10^7 cells) was introduced into HEK 293 cells (10^7 cells per lane), and the lysate was prepared from unstimulated cells. The lysate from each culture plate was mixed in one tube, and then divided into three. Association of ACK with Grb2 was detected by immunoprecipitation with a control rabbit IgG (1 μg), an anti-ACK antibody (sc-323, 1 μg), or an anti-ACK antibody (sc-323, 1 μg) plus an epitope peptide (sc-323P, 3 μg), and subsequent immunoblotting with an anti-Grb2 antibody (MS-20-3, 5 μg/ml). The amounts of precipitated ACK were also determined by immunoblotting with an anti-ACK antibody (sc-323, 1 μg/ml). (B) Increased association of ACK with Grb2 in EGF-stimulated HEK 293 cells. pCMV5-hACK (20 μg for 10^7 cells) was introduced into HEK 293 cells (10^7 cells per lane). The cells were stimulated with EGF (100 ng/ml) for 1 min at 37°C or left unstimulated. Association of ACK with Grb2 was compared between unstimulated (control) and EGF-stimulated (+EGF) cells as in (A). The total amounts of ACK within the lysates were also determined by immunoblotting with an anti-ACK antibody (sc-323, 1 μg/ml). (C) Association of Shc with Grb2 in EGF-stimulated HEK 293 cells. HEK 293 cells (10^7 cells per lane) were stimulated with EGF (100 ng/ml) for 1 min at 37°C or left unstimulated. Association of Shc with Grb2 was compared between unstimulated (control) and EGF-stimulated (+EGF) cells by immunoprecipitation with a control rabbit IgG (1.5 μg) or an anti-Shc antibody (06-203, 1.5 μg), and subsequent immunoblotting with an anti-Grb2 antibody (MS-20-3, 5 μg/ml). (D) Association of ACK with Grb2(ΔSH2). pCMV5-hACK (20 μg for 10^7 cells) and an expression plasmid for Grb2(ΔSH2) (pCMV5-hGrb2(ΔSH2), 20 μg for 10^7 cells) were introduced into HEK 293 cells (10^7 cells per lane). Association of ACK with endogenous Grb2 as well as Grb2(ΔSH2) in unstimulated cells was detected as in (A).

4. Discussion

Although the temperature shift-down and the hypertonic shock induce no obvious phenotypic change in HEK 293 cells within 0.5 or 1 h, our observation that they cause tyrosine phosphorylation of ACK may be potentially important because the regulation of protein phosphorylation by these kinds of stress seems novel and unique to ACK. The temperature decrease to 25°C as well as the hyperosmolarity generally show no effect on the activity of tyrosine kinases. In fact, we observed no change of the tyrosine phosphorylation level of c-Src in HEK 293 cells in response to these stimuli (Fig. 1C, and data not shown). Also, it should be noted that the temperature shift to 42°C did not affect the ACK tyrosine phosphorylation (Fig. 1A, and data not shown), irrespective of effective activation of the SAPK/JNK pathway at 42°C [28]. Thus, a unique mechanism to regulate the tyrosine phosphorylation level of ACK, which is distinct from the regulatory mechanism of the SAPK/JNK pathway, may exist. It should be clarified whether induction of the ACK tyrosine phosphorylation is mediated by Cdc42 although it is not fully understood whether Cdc42 and Rac GTPases are involved in the case of the stress-induced SAPK/JNK activation. Moreover, it remains obscure whether ACK is tyrosine phosphorylated by itself (autophosphorylation) or by a distinct tyrosine kinase, whose activity is enhanced by the temperature shift-down or the hyperosmolarity. It may also be possible that a temperature- or osmolarity-sensitive tyrosine phosphatase is impaired following the stimulation, thereby increasing the tyrosine phosphorylation level of ACK. Future experiments should reveal the physiological significance of cold- and hyperosmotic-induction of ACK tyrosine phosphorylation as well as the biological role of ACK in vivo.

Additionally, EGF was found to be capable of stimulating the tyrosine phosphorylation of ACK at 37°C. The results that ACK forms a complex with Grb2 through the interaction with the Grb2 SH3 domain may provide an insight into the regulatory mechanism of ACK tyrosine kinase by the EGF receptor. It may be possible that the integration of ACK into a signaling particle comprised of adaptor proteins and the EGF receptor may cause the translocation of ACK to the close vicinity of the activated EGF receptor within the plasma membrane. Although it remains obscure whether the association between ACK and Grb2 is direct, it is possible that two class I (amino acid residue 685–688 and 834–837) and two class II (791–794 and 800–803) PXXP SH3-binding motifs (the proline-rich domains) [29] within ACK protein may serve as the binding site of Grb2. Alternatively, a still unknown molecule may bind to a specific region of ACK, such as the SH3 domain that resides contiguous to the Cdc42-binding domain, thereby linking ACK and Grb2.

As shown in Fig. 4B, the association of ACK with Grb2 was increased following EGF stimulation. In the case of the Ras-stimulating pathway, the association between Grb2 and mSos through the SH3 domain/proline-rich domain interaction was first reported to be constitutive [30-33]. However, it has recently been shown that the Grb2/mSos association is also regulated by intracellular signals. For instance, stimula-

tion of the EGF receptor [34] or the T cell receptor complex [35,36] increased the association of Grb2 with mSos. The enhancement of the complex formation of ACK with Grb2 following EGF stimulation may be due to a conformational change of the complex upon the binding of Shc to Grb2 (Fig. 4C).

Recently, a gene product designated WASP, which is related to the Wiskott-Aldrich syndrome, was reported to physically associate with Nck adaptor protein through the SH3 domain [37]. Interestingly, WASP contains the Cdc42/Rac-interacting motif, being another target of Cdc42/Rac family GTP-binding proteins [16,38]. Therefore, like ACK tyrosine kinase, WASP may be regulated by both direct interaction with Cdc42·GTP and a signal through a tyrosine kinase/adaptor cascade. The convergence of signals mediated by small GTP-binding proteins or adaptor proteins may become a novel type of the cross-talk among the intracellular signal transduction pathways.

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