Novel function of Chat in controlling cell adhesion via Cas-Crk-C3G-pathway-mediated Rap1 activation

Akira Sakakibara[1,*,§], Yusuke Ohba[2], Kazuo Kurokawa[2], Michiyuki Matsuda[2] and Seisuke Hattori[1,‡]

[1]Division of Biochemistry and Cellular Biology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan
[2]Department of Tumor Virology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan
*Present address: Department of Cell Biology, University of Virginia, Charlottesville, VA 22908, USA
‡Present address: Division of Cellular Proteomics, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108-8639, Japan
§Author for correspondence (e-mail: as6by@virginia.edu)

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Summary

Chat (Cas/HEF1-associated signal transducer) is a novel signaling molecule with an N-terminal SH2 domain and C-terminal Cas/HEF1 association domain that is implicated in the regulation of cell adhesion. The Cas/HEF1 association domain also shows sequence similarity with guanine nucleotide exchange factors for Ras family small GTPases. In this study, we found significant activation of Rap1 in Chat-overexpressing cells. Myr-Chat, a membrane-targeted form of Chat, activated Rap1 more efficiently. Interestingly, Chat and Cas synergistically activated Rap1. Certain Cas, Crk or C3G mutants suppressed Rap1 activation by Chat. We also confirmed the ternary complex formation consisting of Chat, Cas and Crk. Thus, it is likely that Chat-induced Rap1 activation was mediated by upregulation of the Cas-Crk-C3G signaling pathway rather than direct guanine nucleotide exchange factor activity of Chat. We further demonstrated that Myr-Chat expression induced cell periphery spreading and cell shape branching and that this activity also depended on the Cas-Crk-C3G pathway and Rap1 activity. Moreover, expression of Myr-Chat enhanced integrin-mediated cell adhesion. Taken together we propose a novel role for the Chat-Cas complex in controlling cell adhesion via the activation of Rap1.

Key words: Cas, Cell adhesion, Chat, Guanine nucleotide exchange factor, Rap1, Small GTPase

Introduction

Cell adhesion to the extracellular matrix is essential for diverse biological processes such as cell growth, survival, differentiation and migration (Hynes, 1992; Schwartz et al., 1995; Lauffenburger and Horwitz, 1996). In various cell types, integrin receptors serve as a linker for cell-matrix adhesion. Integrin-mediated adhesion to the extracellular matrix activates a subset of intracellular signaling pathways, which alters the gene expression pattern and cytoskeletal structures. On the other hand, the adhesive property of integrins is also regulated by an inside-out manner upon certain extracellular stimuli. However, the molecular basis underlying these regulatory mechanisms remains elusive.

It has been well characterized that Src family tyrosine kinases and Cas family docking proteins play a pivotal role in the integrin-mediated signaling pathways (Hanks and Polte, 1997; O'Neill et al., 2000; Bouton et al., 2001). Cas is a multivalent docking protein that transduces the adhesion-dependent tyrosine-phosphorylation signal. At the submembranous region of integrin adhesion sites, activated focal adhesion kinase (FAK) forms a complex with Cas and Src family kinases, which induces tyrosine-phosphorylation of the Cas substrate domain (Schlaepfer et al., 1997; Ruest et al., 2001). The Src homology 2 (SH2) domain of Crk binds to tyrosine-phosphorylated residues of the Cas substrate domain (Sakai et al., 1994), recruiting downstream Crk SH3-domain-binding effectors toward Cas (Tanaka et al., 1994; Hasegawa et al., 1996).

It has also been shown that various small GTPases function in integrin-mediated signaling pathways. Rap1 regulates inside-out modulation of integrin function (Katagiri et al., 2000; Reedquist et al., 2000; Caron et al., 2000; Ohba et al., 2001; Bos et al., 2001). R-Ras is also involved in the inside-out activation of integrins (Zhang et al., 1996). Ras activates the extracellular signal-regulated kinase (ERK) downstream of integrin adhesion (Clark and Hynes, 1996). Two major effectors of Crk are C3G and DOCK 180, which, respectively, activate the small GTPases Rap1 and Rac1 (Gotoh et al., 1995; Kiyokawa et al., 1998). Rac1 activates the c-Jun N-terminal kinase (JNK) cascade that transmits the integrin-mediated mitotic signal to the nucleus (Dolfi et al., 1998) and promotes cell migration through actin filament reorganization (Klemke et al., 1998).

Recently, we have identified a novel signaling molecule, Chat (Cas/HEF1-associated signal transducer), as a binding partner of the Cas family proteins, Cas and HEF1 (Sakakibara and Hattori, 2000). Chat contains an SH2 domain and is phosphorylated by mitogen-activated protein (MAP) kinase, suggesting that Chat integrates signals from tyrosine kinases and MAP kinase. We also showed that Chat and Cas were associated with each other through their C-terminal domains. Chat is a member of a structurally related protein family that

consists of NSP1 (Lu et al., 1999), AND-34 (Cai et al., 1999)/BCAR3 (van Agthoven et al., 1998)/NSP2 and Chat/SHEP1 (Dodelet et al., 1999)/NSP3. All of the family proteins are reported to be associated with Cas. Besides the Cas-binding activity, the C-terminal domain of Chat family proteins shares weak amino-acid sequence similarity with the catalytic domain of guanine nucleotide exchange factors (GEFs) (reviewed in Overbeck et al., 1995) for Ras family small GTPases (Dodelet et al., 1999; Gotoh et al., 2000). Overexpression of AND-34 was reported to activate small GTPases, RalA, Rap1 and R-Ras, which was inhibited by co-expression of Cas and AND-34 (Gotoh et al., 2000). By contrast, Dodelet et al. reported that SHEP1, an alternatively transcribed isoform of Chat, binds to R-Ras and Rap1 without activating them (Dodelet et al., 1999). Thus, the function of Chat family members in the regulation of Ras family proteins still remains unclear.

Therefore, to further investigate the Chat function, we examined the effect of Chat expression on Ras family GTPases. The expression of Chat induced a significant activation of the small GTPase Rap1, but not H-Ras, R-Ras or RalA, in 293T cells. The membrane-targeted form of Chat (Myr-Chat) showed higher activity in Rap1 activation. Interestingly, co-expression of Cas enhanced the activation of Rap1, and dominant-negative mutants of Cas, Crk or C3G counteracted the effect of Chat. These results suggest that the Chat-dependent Rap1 activation was not mediated by the direct effect of Chat on Rap1 but was rather dependent on the Chat-Cas interaction and downstream Crk-C3G pathway. We also found that expression of Myr-Chat in NIH3T3 cells induced cell protrusions at the cell periphery, which was frequently accompanied by branched cell morphology. This cell-shape-converting activity also required the Cas-Crk-C3G signaling pathway and Rap1 function. Furthermore, Myr-Chat expressing 293T cells showed an increased adhesion to the fibronectin matrix. These results suggest that the Chat-Cas complex plays a role in coupling certain tyrosine-kinase-derived signals to modulate cell adhesion via Rap1 activity.

Materials and Methods

Materials and cells

Expression plasmids for FLAG-tagged Chat (Sakakibara and Hattori, 2000), HA-tagged Cas (Nakamoto et al., 1997), Myc-tagged Crk (Tanaka et al., 1993), C3G dCD (Tanaka et al., 1997) and FLAG-tagged small GTPases (Ohba et al., 2000b) were described previously. The expression construct for HA-tagged Cas-C-half ($Cas_{425-874}$) was described above (Sakakibara and Hattori, 2000). pCAX-Myr-Chat, a membrane-targeted Chat-expressing plasmid, was constructed by adding the v-Src derived myristoylation signal (Kohn et al., 1996) to the N-terminus and the FLAG epitope to the C-terminus, followed by subcloning into an expression vector pCAGGS (Niwa et al., 1991). pIRES-EGFP, a derivative of pCAGGS carrying enhanced green fluorescent protein (EGFP) with a preceding internal ribosomal entry site (IRES) downstream of the multiple cloning site was used for analyzing the morphology of transfected cells. Anti-Chat SH and anti-Crk 3A8 were previously described (Sakakibara and Hattori, 2000; Matsuda et al., 1993). Anti-FLAG M2, anti-HA 3F10 and anti-Cas were purchased from Sigma, Roche and Transduction Lab., respectively. 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. NIH3T3 cells were cultured in DMEM containing 10% calf serum.

Pull-down assay for GTP-bound small GTPases

The relative amount of GTP-bound small GTPases was determined by Bos's pull-down method with the following modifications (Franke et al., 1997; Ohba et al., 2000a). The following binding domains for Ras family GTPases (RBDs) fused to glutathione *S*-transferase (GST) were used for GTP-bound GTPase pull-down; Raf-RBD for H-Ras and R-Ras, RalGDS-RBD for Rap1 or RalGDS-RBD for RalA. Semi-confluent 293T cells on poly-L-lysine-coated dishes were transiently transfected with expression plasmids for FLAG-tagged small GTPases by using Lipofectamine 2000 reagent (Life Tech.). 30 hours after transfection, cells were lysed with ice-cold PD buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin), and samples were kept on ice for 10 minutes. After clarification by centrifugation, GTP-bound small GTPases were pulled down with GST-RBD fusion-protein-adsorbed glutathione-Sepharose beads (Amersham Pharmacia Biotech.) for 30 minutes on ice. The complex were washed three times with PDW buffer (PD buffer containing 0.5% Triton X-100) and then eluted with sample buffer for SDS-polyacrylamide gel electrophoresis. Immunoblotting of the samples was carried out as previously described (Sakakibara and Hattori, 2000).

Co-immunoprecipitation assay

293T cells were transiently transfected with the indicated expression constructs using a calcium phosphate method. 36 hours after transfection, cells were lysed in ice-cold IP buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin), followed by centrifugal clarification. Samples were immunoprecipitated with anti-Cas or anti-Crk 3A8 mouse monoclonal antibodies by using protein A Sepharose (Amersham Pharmacia Biotech.). Co-immunoprecipitated Chat was detected by anti-Chat immunoblotting.

Immunofluorescence microscopy

Indirect immunofluorescence microscopy was carried out as described above (Sakakibara and Hattori, 2000). NIH3T3 cells on glass cover slips were transfected with pCAX-Chat or pCAX-Myr-Chat using Lipofectamine Plus reagent (Life Tech.) followed by 30 hours of incubation. After fixation of the cells, anti-FLAG was used as a primary antibody for detection of Chat or Myr-Chat. Fluorescein (FITC)-conjugated goat anti-mouse IgG (Jackson Lab.) or Texas-Red-conjugated phalloidin (Molecular Probes) was, respectively, used for visualizing the anti-Chat immune complex or the actin filament.

Cell area estimation

NIH3T3 cells plated on 0.5 μg/ml fibronectin-coated 35 mm glass base dish (Iwaki) were transiently transfected with pIRES-EGFP vector-based expression constructs using Lipofectamine 2000 reagent. 30 hours after transfection, fluorescent images of EGFP-expressing cells were recorded using a Zeiss Axiovert microscope (Carl Zeiss) with a cooled CCD camera (Roper Scientific), controlled by MetaMorph2 software (Universal Image). The cell area of each GFP-positive cell was estimated by measuring the EGFP-fluorescence-emitting region using MetaMorph2 software. For co-transfection experiments, cells were transfected with a five-fold amount of a mutant protein expressing plasmid together with pIRES-EGFP-Myr-Chat.

Cell adhesion assay

In this study, we have developed a novel system for cell adhesion

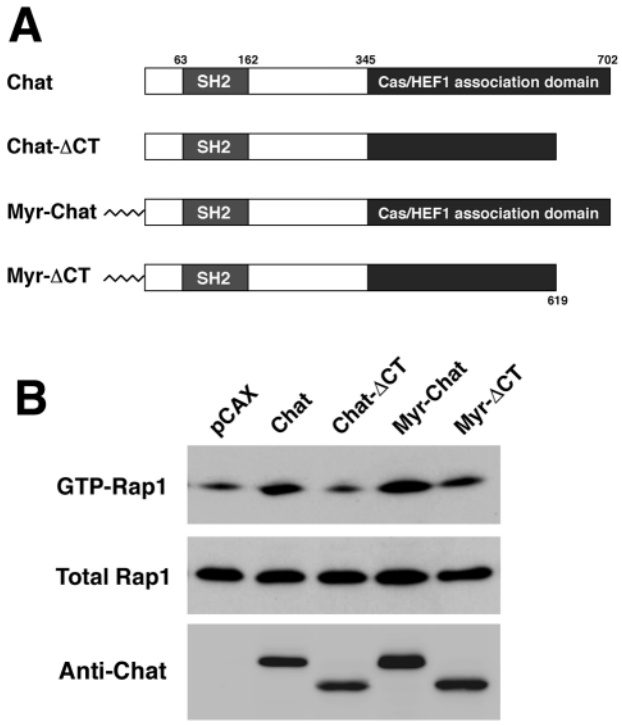

Fig. 1. Rap1 activation induced by overexpression of Chat. (A) A diagram of Chat variants used in this study. We used an N-terminally myristoylated membrane-targeted form of Chat (Myr-Chat) to evaluate the activation status of Chat. C-terminal deletion mutants (Chat-ΔCT and Myr-ΔCT) lacked the Cas association activity. (B) 293T cells were transiently transfected with the indicated Chat-related expression plasmid together with small GTPase Rap1. After 30 hours, the activation level of Rap1 was compared by a pull-down assay. The amount of pulled-down GTP-bound Rap1 (GTP-Rap1), expression levels of transfected Rap1 (Total Rap1) and Chat-derivatives (Anti-Chat) is shown. Overexpression of Chat increased the GTP-bound Rap1 compared with the control pCAX vector transfectant. Myr-Chat activated Rap1 more effectively than wild-type Chat. Chat-ΔCT and Myr-ΔCT abolished the Rap1 activation competence.

assays using transiently transfected 293T cells. An expression plasmid for EGFP was always cotransfected, and the adhesion of transfected cells was quantified by measuring the EGFP-derived fluorescence. Briefly, 293T cells were co-transfected with 1.5 μg pCAX-EGFP and 3.5 μg plasmid to be examined as described in the small GTPase pull-down assay section. 24 hours after transfection, cells were serum starved for 6 hours at 37°C in an adhesion medium (DMEM supplemented with 0.5% bovine serum albumin). Microwell culture plates (96 well) were coated with PBS containing 0.2 μg/ml fibronectin (Sigma) for 2 hours at 37°C, blocked for 1 hour with DMEM supplemented with 3% bovine serum albumin followed by three washes with the adhesion medium. The cells were dispersed in PBS containing 0.01% trypsin, followed by adding 10 μg/ml trypsin inhibitor and washing with PBS. After re-suspension in the adhesion medium, 2×10^5 cells were added into each well and allowed to adhere for 30 minutes at 37°C. EGFP-derived fluorescence was measured as the total loaded cell-derived fluorescence (Ft) using a Fluorimager (Molecular Dynamics). To remove non-adherent cells, the wells were washed four times with the adhesion medium. Remaining fluorescence was measured as the adherent cell-derived fluorescence (Fa). The percentage of adherent cells was calculated from the ratio of Fa/Ft.

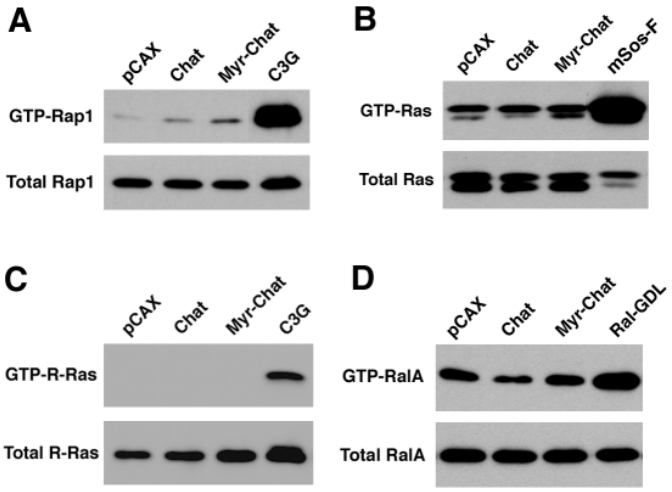

Fig. 2. Effect of Chat overexpression on Ras family small GTPase activities. Activation levels of the small GTPases in Chat-overexpressing 293T cells were examined by a pull down assay as in Fig. 1. (A) Overexpression of Chat or Myr-Chat induced Rap1 activation. The activation level was low compared with a positive control Rap1 GEF, C3G. Neither Chat nor Myr-Chat increased the amount of GTP-bound H-Ras (B), R-Ras (C) and RalA (D) in similar experiments to A. mSos-F, a membrane-targeted form of mSos, C3G or RalGDL was used as a positive control for H-Ras, R-Ras or RalA activation.

Results

Chat overexpression activates Rap1 in 293T cells

In our previous study, we identified Chat as an adaptor protein involved in the Cas signaling pathway (Sakakibara and Hattori, 2000). Besides this unique property, the Chat C-terminal Cas/HEF1 association domain also shows a weak sequence similarity to the catalytic domain of GEFs for Ras family small GTPases (Dodelet et al., 1999; Gotoh et al., 2000). Therefore, we examined whether Chat functions as a GEF for certain small GTPases using the various Chat constructs illustrated in Fig. 1A.

First, we found that the expression of Chat caused a slight increase in GTP-bound Rap1 in 293T cells (Fig. 1B) when the active Rap1 was analyzed using a modified pull-down assay originally developed by Bos' group (Franke et al., 1997; Ohba et al., 2000a) (see Materials and Methods). By measuring the intensity of the corresponding bands, a statistically significant increase in GTP-bound Rap1 (2.4±1.2 times, n=5, P<0.05) was reproducibly observed in Chat overexpressing cells compared with control pCAX-transfected cells. An N-terminally myristoylated Chat variant (Myr-Chat) showed higher activity in Rap1 activation (3.7±1.4 times, n=5, P<0.01). To elucidate whether the Chat C-terminal Cas/HEF1 association (GEF-like) domain was required for the activation of Rap1, we examined the effect of C-terminally truncated mutants, Chat-ΔCT and Myr-Chat-ΔCT, on Rap1 activation. Both mutants did not show statistically significant activation of Rap1 (1.2±0.4 times, n=3, P<0.5 and 1.3±0.2 times, n=3, P<0.1, respectively), indicating that the function of the Chat C-terminal domain was essential for the Rap1 activation.

We next analyzed the specificity of Chat activity toward several Ras family small GTPases (Fig. 2). As shown in Fig.

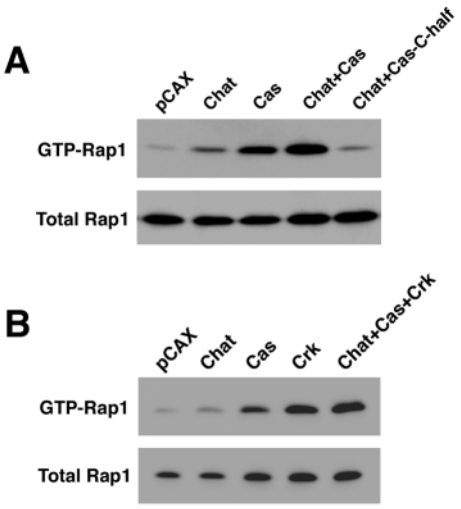

Fig. 3. Overexpression of the Chat-Cas-Crk pathway member activates Rap1. (A) Overexpression effect of Chat and Cas on Rap1 activation was examined by a pull-down assay of GTP-bound Rap1 as in Fig. 1. Overexpression of Cas increases the amount of GTP-bound Rap1 like Chat. Co-expression of Cas with Chat enhanced the Rap1 activation (Chat+Cas), but the C-terminal portion of Cas inhibited it (Chat+Cas-C-half). (B) Rap1 activation by Chat, Cas or Crk overexpression. Overexpression of Crk showed the strongest Rap1 activation. Enhanced Rap1 activation by co-expression of Chat and Cas with Crk was hard to detect because of the robustness of the Crk-induced Rap1 activation.

2A, overexpression of Chat or Myr-Chat again induced Rap1 activation, although the activation level was rather low compared with that caused by C3G. Chat did not significantly activate other Ras family small GTPases, H-Ras, R-Ras and RalA (Fig. 2B-D). Taken together, the results from several independent experiments established that Chat reproducibly activated Rap1 but not other GTPases (Fig. 1B, Fig. 2; data not shown). We also observed a slight decrease (35% decrease, n=3, P<0.005; Fig. 2D; data not shown) of GTP-bound RalA in Chat- but not Myr-Chat-expressing cells, although the meaning is unclear.

Functional Cas-Crk-C3G signaling pathway is required for the Chat-induced Rap1 activation

Interestingly, we further found that the overexpression of Cas also activates Rap1 and that co-expression of Chat and Cas caused enhanced activation of Rap1 (Fig. 3A). By contrast, co-expression of the Cas C-terminal portion (Cas-C-half; amino-acid residues 425-874), which lacked both SH3 and substrate domains of Cas, inhibited the Chat-mediated Rap1 activation. Overexpression of Crk also robustly activated Rap1 with higher efficiency (Fig. 3B). These results raised the possibility that the Chat-induced Rap1 activation was mediated by Chat-Cas-interaction inducing the upregulation of the downstream Crk-C3G pathway rather than by intrinsic GEF activity of Chat. Since Crk activates Rap1 rather strongly, triple expression of Crk, Cas and Chat did not further increase the activation level (Fig. 3B).

Therefore, we next analyzed the effect of several Cas mutants on Rap1 activation. Cas is a multivalent docking protein that consists of an SH3 domain, substrate domain (SD), Src-binding domain and Chat association domain (Fig. 4A) (see Nakamoto et al., 1997; Sakakibara and Hattori, 2000; Bouton et al., 2001). The Cas SH3 domain interacts with a number of signaling molecules such as, FAK, Pyk2, PTP-1B, PTP-PEST and C3G. Cas SD contains multiple tyrosine phosphorylation sites that serve as docking sites for Crk SH2 domain. As shown in Fig. 4B, deletion of Cas SD (CasΔSD) almost completely impaired activation of Rap1 (1.0 fold, n=4). As expected, CasΔSD was not tyrosine phosphorylated at all and did not bind Crk (Fig. 4C), which may be the reason for its inability to activate Rap1. Surprisingly, Cas ΔSCB, lacking the region essential for Src binding and Chat association, still retained substantial tyrosine phosphorylation level, Crk association and Rap1-activating activity (2.0 fold, n=4). These results revealed a crucial role of the Cas substrate domain and its interaction with Crk in the Cas-induced

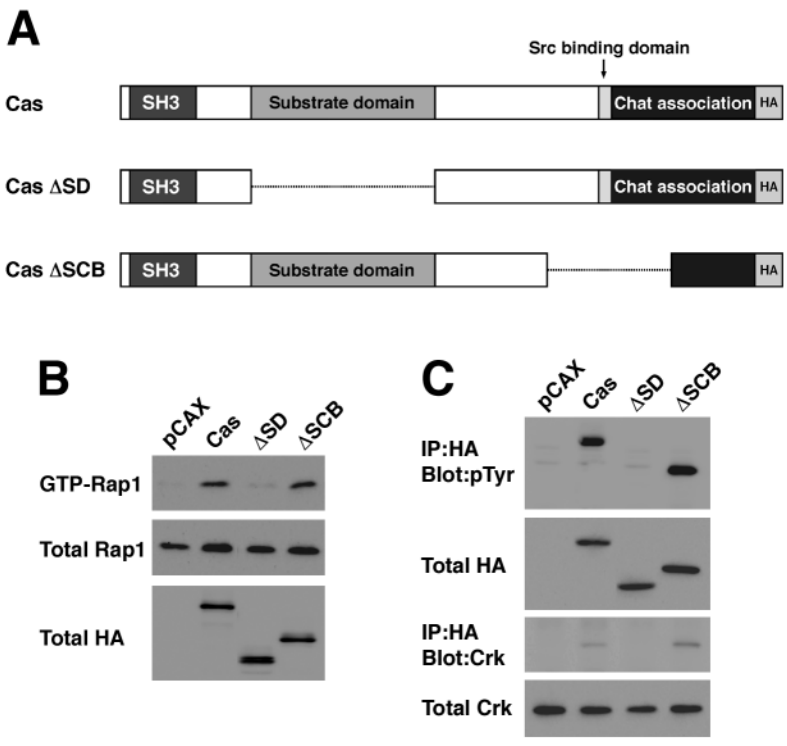

Fig. 4. Deletion effect of Cas domain structure on Cas-induced Rap1 activation. (A) Schematic representation of Cas variants used for the evaluation of Rap1 activation competence. (B) Overexpression effect of Cas and its deletion mutants on Rap1 activity was examined as in Fig. 1. Cas overexpression increases the amount of GTP-bound Rap1 compared with control pCAX transfectant. Cas ΔSD failed to activate Rap1, whereas Cas ΔSCB substantially retained the activity. (C) Tyrosine phosphorylation and Crk interaction of Cas mutants. 293T cells were transfected with a Cas derivative expression plasmid together with Crk. After immunoprecipitation with anti-HA antibody, the tyrosine phosphorylation levels of Cas proteins and co-immunoprecipitated Crk in the immunoprecipitates were analyzed.

Rap1 activation. Therefore, we used Cas ΔSD mutant in the following experiments as an interfering mutant for Chat-mediated Rap1 activation.

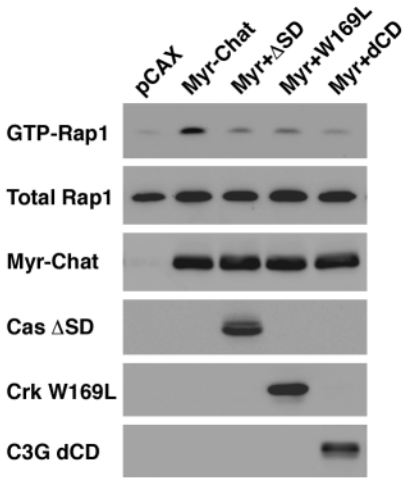

Fig. 5. Suppression of Myr-Chat-mediated Rap1 activation by dominant-negative mutants of Cas, Crk or C3G. 293T cells were co-transfected with Myr-Chat and Cas, Crk or C3G mutant expression plasmid. After 30 hours, the Rap1 activation level of these transfected cells was examined. Co-expression of Cas ΔSD (Myr+ΔSD), Crk W169L (Myr+W169L) or C3G dCD (Myr+dCD) effectively suppressed the Rap1 activation induced by Myr-Chat.

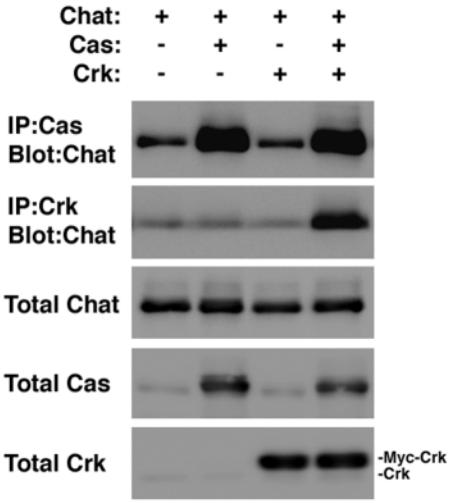

Fig. 6. Chat is associated with the Cas-Crk signaling complex. The association of Chat with Cas or Crk was examined by a co-immunoprecipitation assay. 293T cells were transfected with (+) or without (–) expression plasmids for Chat, Cas or Crk. Transfected cell lysates were immunoprecipitated with anti-Cas or anti-Crk antibodies; co-immunoprecipitated Chat was detected by an anti-Chat immunoblot. The expression level of Chat, Cas and Crk was analyzed by total lysate immunoblot of each protein. The different mobilities of endogenous Crk (Crk) and Myc-tagged Crk (Myc-Crk) are shown in the right side of the panel. The amount of Chat co-immunoprecipitated with Cas was not affected by Crk expression level. In Chat and Crk co-expressing cells, the amount of Chat co-immunoprecipitated with Crk was not increased. When Cas was expressed together with Chat and Crk, a marked increase of Chat in Crk immunoprecipitate was observed.

To address whether the Cas-Crk-C3G signaling pathway indeed mediates the Chat-induced Rap1 activation, we expressed dominant-negative mutants of Cas, Crk or C3G together with Myr-Chat and evaluated the effect of these mutants on the activation of Rap1 (Fig. 5). As expected, co-expression of Cas ΔSD (Myr+ΔSD) significantly suppressed the Myr-Chat-induced Rap1 activation. Furthermore, Crk W169L (Myr+W169L), a loss of function mutant of Crk SH3 domain (Tanaka et al., 1993), or C3G dCD (Myr+dCD), C3G devoid of its catalytic domain (Tanaka et al., 1997), also interfered with the Rap1 activation induced by Myr-Chat. A similar inhibitory effect of these mutants on Rap1 activation was observed when Chat instead of Myr-Chat was used (data not shown). Thus, the functional Cas-Crk-C3G signaling pathway is required for Chat-mediated upregulation of Rap1.

We then tried to identify a signaling complex consisting of Chat, Cas, Crk and C3G by a co-immunoprecipitation assay. As shown in Fig. 6, Chat was recovered in anti-Cas-immunoprecipitates from Chat-expressing 293T cells, and the amount of Chat was greatly increased by co-expression of Cas. In the control experiment, Chat was not detected at all in immunoprecipitates with a control antibody (data not shown). Increased expression of Crk did not affect the amount of Chat in the Cas immune complex. Recovery of Chat in anti-Crk immunoprecipitates of Chat-overexpressed cells was rather low, and its recovery greatly enhanced in Chat-Cas-Crk triple-transfected cells. These results clearly indicate a ternary complex formation consisting of Chat, Cas and Crk and provide substantial support for the Chat-Cas-Crk-C3G signaling pathway inducing Rap1 activation.

Overexpression of Cas did not significantly increase the Chat recovery in Crk immunoprecipitates. Assuming that the Chat-Crk interaction is mediated by Cas, excess Cas might inhibit the recovery of Chat in Crk-immunoprecipitates depending on the molar ratios of Chat, Cas and Crk. Finally, we examined a possible interaction between Chat and C3G by using a similar immunoprecipitation method. However, it was hard to detect a significant association to form a quaternary complex containing Chat and C3G (data not shown).

Membrane-targeted Myr-Chat expression induces cell periphery spreading and cell shape branching

Recent reports from several laboratories implicate Rap1 in the inside-out modulation of integrin adhesion (Katagiri et al., 2000; Reedquist et al., 2000; Caron et al., 2000; Ohba et al., 2001), and cell adhesion properties often affect the cell morphology. Therefore, we studied the effect of the membrane-targeted form of Chat (Myr-Chat) on cell morphology. Intracellular localization of Chat or Myr-Chat was visualized by anti-FLAG immunofluorescence together with phalloidin staining of actin filaments to illustrate the cell shape. Chat was distributed throughout the cytoplasm, and no apparent cell morphological alteration was observed compared with non-transfected cells in the same field (Fig. 7A,B) (data not shown). Myr-Chat-derived fluorescence signal was mainly from the plasma membrane and intracellular vesicle-like structures (Fig. 7C,E). Interestingly, Myr-Chat-expressing cells frequently showed highly spread cell periphery and branched cell shape (Fig. 7C-E). This morphology was obviously distinct from a typical pancake-like spreading image often induced by

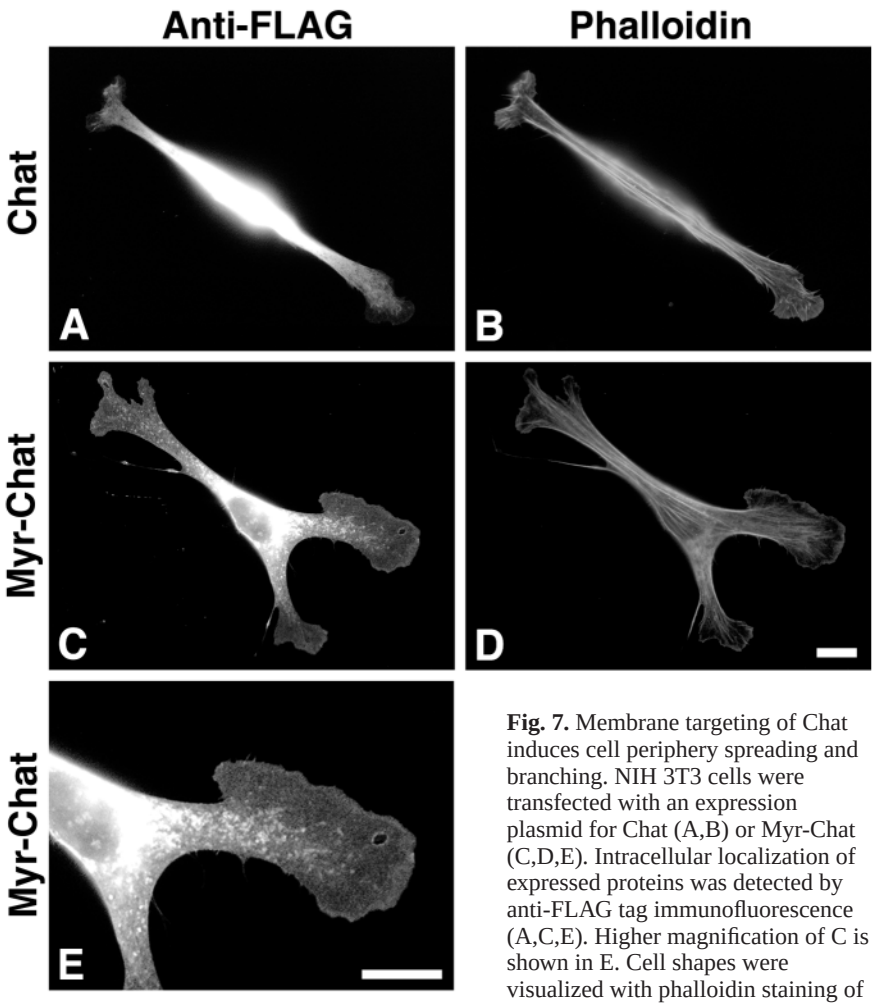

Fig. 7. Membrane targeting of Chat induces cell periphery spreading and branching. NIH 3T3 cells were transfected with an expression plasmid for Chat (A,B) or Myr-Chat (C,D,E). Intracellular localization of expressed proteins was detected by anti-FLAG tag immunofluorescence (A,C,E). Higher magnification of C is shown in E. Cell shapes were visualized with phalloidin staining of the actin filament (B,D). Chat was detected mainly in the cytoplasm (A). Myr-Chat was localized at the plasma membrane and intracellular vesicle like spots (C,E). Myr-Chat but not Chat expression induced spreading of cell periphery, which was frequently accompanied by branched cell morphology (B,D). Bars, 10 μm.

constitutive active Rac (Nobes and Hall, 1995; Kiyokawa et al., 1998). Co-expression of Cas with Chat does not alter the morphology of the cells, even though it activated Rap1 robustly (data not shown).

To quantify the morphological change, we used the pIRES-EGFP-based expression system that could simultaneously express a gene of interest with EGFP from a single transcript and visualize the transfected live cells. The area of each transfected cell was measured as the size of the fluorescence-emitting region. Under our experimental conditions, most of control pIRES-EGFP-transfected NIH3T3 cells showed spindle-like cell shape (Fig. 8). As shown in Fig. 7, Myr-Chat-expressing cells frequently showed cell protrusions and branched morphology. The quantified cell area values of these EGFP-positive cells were represented as histograms (Fig. 8. right panels). The cells expressing Myr-Chat showed substantially increased cell area values compared with control cells. Overexpression of wild-type Chat did not induce a significant increase. As in the case of Rap1 activation, a C-terminal deletion of Myr-Chat (Myr-ΔCT) completely inactivated the cell-area-enlarging activity.

We further examined the effect of dominant-negative mutants of Cas, Crk and Rap1 on Myr-Chat-induced cell area enlargement (Fig. 9). In good agreement with the inhibitory effect on Myr-Chat-induced Rap1 activation (Fig. 5), Cas ΔSD, Crk W169L, and C3G dCD effectively suppressed the spreading and branching of cell periphery and cell area enlargement induced by Myr-Chat. N17 Rap1, a dominant-negative mutant of Rap1, also interfered with the Myr-Chat-induced cell shape conversion. Under these conditions, the membranous localization of Myr-Chat was not affected by co-expression of these mutants (data not shown). These results indicate that the morphological change of Myr-Chat-expressing cells is mediated by the Cas-Crk-C3G signaling pathway, leading to Rap1 activation.

Myr-Chat expression modulates integrin-mediated adhesion of 293T cells

To gain insight into the cellular mechanism underlying the morphological effect of Myr-Chat, we studied the adhesion of cells to fibronectin as Rap1 activity had been implicated in cell adhesion. We used the dishes coated with low concentrations of fibronectin (0.2 μg/ml) to make the difference in cell adhesion stand out. The cells expressing Myr-Chat showed 50% greater cell adhesion when compared to cells transfected with a control vector pCAX (Fig. 10). Under the same conditions, we could not detect a significant increase in cell adhesion when the wild-type Chat was overexpressed. Myr-Chat-ΔCT also was ineffective, indicating the essential role of the Cas/HEF1 association domain in modulating the cell adhesion. These results suggest a function of Chat-Cas complex in regulation of cell adhesion.

Discussion

Chat is a unique adaptor molecule that may link signals from tyrosine kinases to Cas family proteins. However, it is unclear if Chat also serves as a direct GEF for Ras family small GTPases. In the present study, we have shown that Chat and a membrane-targeted form of Chat (Myr-Chat), which is more potent, activate Rap1 (Fig. 1B, Fig. 2A). We further provide evidence that Chat-induced Rap1 activation is mediated by an interaction with a Cas-Crk signaling complex rather than working as a direct GEF for Rap1: (1) overexpression of Cas also activates Rap1 and co-expression of Cas enhances the Chat-induced Rap1 activation (Fig. 3A); (2) certain dominant-negative mutants of Cas, Crk or C3G suppress Rap1 activation by Myr-Chat (Fig. 5); and (3) Chat, Cas and Crk form a ternary

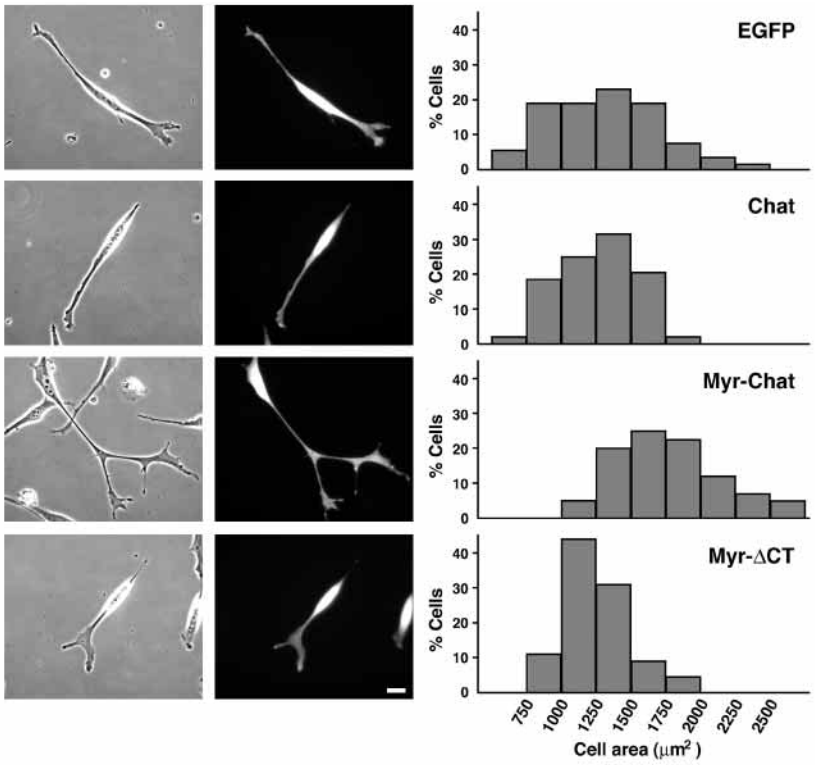

Fig. 8. Myr-Chat-expressing cells show a highly enlarged cell area. NIH 3T3 cells were transfected with indicated pIRES-EGFP-based expression plasmids. Phase-contrast images (left column) and fluorescence images (middle column) of EGFP-positive cells are shown. The cell area of transfected cells was evaluated from the fluorescence-emitting region of EGFP-positive cells and represented as histograms on the right column (EGFP, n=52, MED=1335.6 μm^2; Chat, n=44, MED=1266.3 μm^2; Myr-Chat, n=39, MED=1748.0 μm^2; Myr-ΔCT, n=45, MED=1209.5 μm^2). Membrane-targeted Myr-Chat expression induced cell elongation, which was frequently accompanied by a branched morphology. These cells also showed increased cell area value compared with control pIRES-EGFP-transfectants. Wild-type Chat-transfectants did not show any apparent morphological alterations. Myr-Chat-ΔCT also did not show the cell area enlarging activity. Bar, 10 μm.

complex (Fig. 6). These results suggest that Chat functions as an adaptor for Cas family proteins and transmits signals from tyrosine kinases to factors controlling cell adhesion via downstream Rap1 activity.

The Chat family consists of three closely related members, NSP1 (Lu et al., 1999), AND-34 (Cai et al., 1999)/BCAR3 (van Agthoven et al., 1998)/NSP2 and Chat/SHEP1 (Dodelet et al., 1999)/NSP3, all of which have N-terminal SH2 and C-terminal GEF-like Cas/HEF1 association domains. Identities in amino-acid sequence among these members are around 40%. Recently, Gotoh et al. reported that AND-34 activated RalA, Rap1 and R-Ras (Gotoh et al., 2000). However, overexpression of Cas inhibited the GEF activity of AND-34 toward RalA, which was in striking contrast to our results that co-expression of Cas showed a synergistic effect on Chat-mediated Rap1 activation (Fig. 3A). These differential effects of Cas may reflect the dissimilar mechanism of AND-34 and Chat in the activation of these small GTPases; AND-34 may function as a direct GEF whose activity is inhibited by Cas binding, whereas Chat indirectly activates Rap1 through the Cas-Crk-C3G pathway. However, the effect of Cas on Rap1 activation by AND-34 was not studied; detailed molecular events remain obscure. Bos et al. reported that AND-34 does not activate Rap1 in vitro as an unpublished result (Bos et al., 2001). In another study, Dodelet et al. identified SHEP1 (which is identical to Chat-H, hematopoietic cell-specific Chat) as a downstream target molecule for activated Eph receptors (Dodelet et al., 1999). They also described the binding of the C-terminal half of SHEP1 to R-Ras and Rap1. However, the same region of SHEP1 did not show any GEF activity in vitro (Dodelet et al., 1999).

In our previous studies, we showed that C3G, one of the major downstream effectors of the Cas-Crk pathway, functions as a GEF for Rap1 and R-Ras (Gotoh et al., 1995; Gotoh et al., 1997; Ohba et al., 2000b). We also reported that another Crk effector DOCK 180 promotes the activation of Rac1 (Kiyokawa et al., 1998). Therefore, when Myr-Chat up-regulates the Cas-Crk pathway, it should theoretically activate not only Rap1 but also R-Ras and Rac1. However, we could detect activation of neither R-Ras nor Rac1 in our system (Fig. 2C) (A.S., Y.O., K.K., M.M. et al., unpublished). Two explanations may reconcile this observation. First, as shown in Fig. 2, C3G activation of Rap1 is much higher than that of R-Ras. Second, the small GTPases were overexpressed as GST-fusion proteins in the previous studies, whereas the activation of FLAG-tagged GTPases was measured in this study. In any case, the activation of Rap1 by Chat has been established in this study.

In general, one of the most important roles of SH2 domains is recruiting proteins to specific subcellular locations. Although all Chat family proteins have an SH2 domain at the N-terminal portion, their physiological targets are still unclear except for the information from overexpression systems (Lu et al., 1999; Dodelet et al., 1999). This makes it difficult to study Chat function under physiological conditions. Lipidation signals, such as an N-terminal myristoylation signal and C-terminal CAAX box, have been widely used for membrane targeting of the molecule to mimic the activated state (Aronheim et al., 1994; Gotoh et al., 1995; Hasegawa et al., 1996; Kohn et al., 1996). Therefore, we employed Chat with a myristoylation signal (Myr-Chat) for activated Chat. Membrane targeting of Chat upregulated Rap1 induced cell periphery spreading and branched morphology in NIH3T3 cells and enhanced the integrin-mediated adhesion in 293T cells. Although wild-type Chat also activated Rap1, it affected neither cell morphology nor adhesion. We also observed similar induction of cell

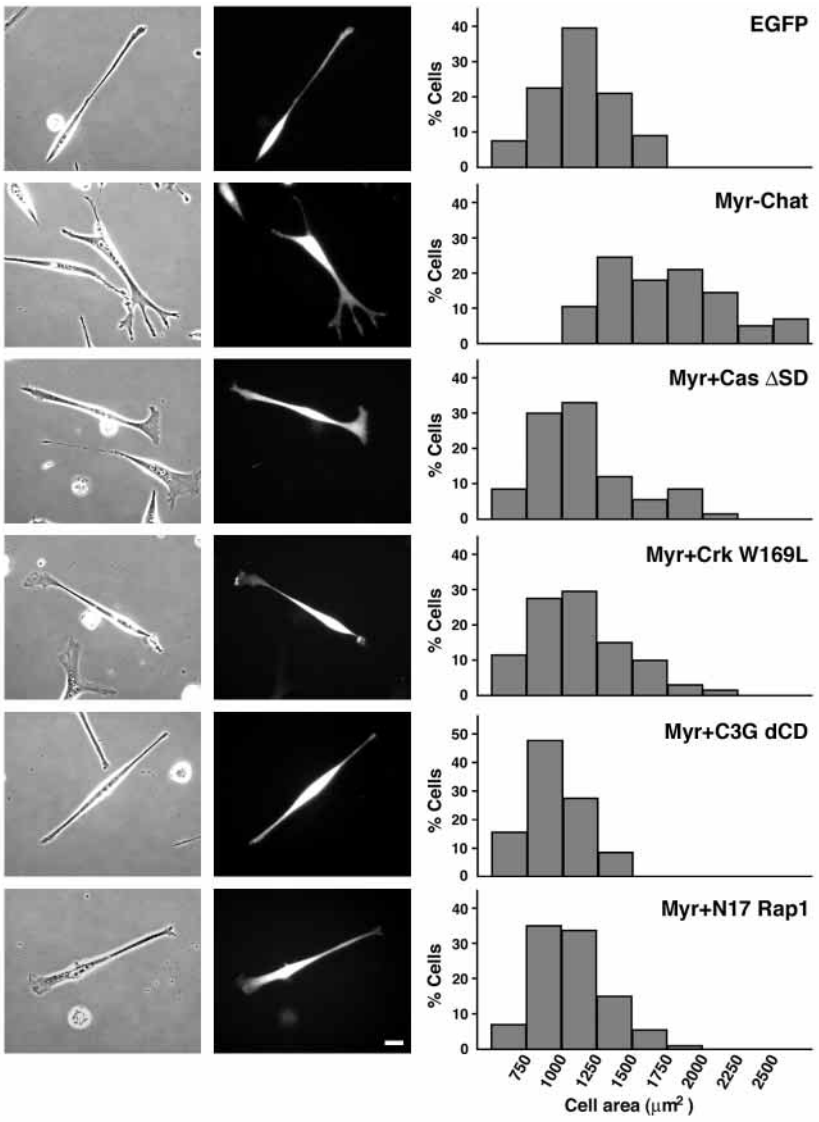

Fig. 9. The morphological effect of Myr-Chat is suppressed by co-expression of dominant-negative mutant of Cas, Crk, C3G or Rap1. NIH 3T3 cells were transfected with pIRES-EGFP (EGFP) or pIRES-EGFP-Myr-Chat (Myr-Chat) together with a plasmid expressing a mutant of Cas (Myr+Cas ΔSD), Crk (Myr+Crk W169L), C3G (Myr+C3G dCD) or Rap1 (Myr+N17 Rap1). Analyses of cell morphology and cell area of transfected cells were performed as in Fig. 8 (EGFP, *n*=66, MED=1118.3 μm^2; Myr-Chat, *n*=57, MED=1699.7 μm^2; Myr+Cas ΔSD, *n*=57, MED=1089.4 μm^2; Myr+Crk W169L, *n*=58, MED=1068.6 μm^2; Myr+C3G dCD, n=57, MED=864.9 μm^2; Myr+N17 Rap1, *n*=65, MED=1063.5 μm^2). Expression of Myr-Chat increased the population of cells with protrusions and branched shape, which caused a cell area value increase. Co-expression of Cas ΔSD, Crk W169L, C3G dCD or N17 Rap1 suppressed the cell-shape-converting effect of Myr-Chat. Bar, 10 μm.

periphery spreading by My-Chat but not by Chat in other cell lines (COS7, PC12) (data not shown). Chat-Cas co-expression even robustly activated Rap1 but was not sufficient for this morphological alteration in these cells (Sakakibara and Hattori, 2000) (data not shown). The requirement for membrane targeting of Chat resembles that of C3G; although both wild-type and membrane-targeted C3G activate Rap1, only the membrane-targeted but not wild-type C3G causes flat reversion of v-Ki-*ras*-transformed cells (Gotoh et al., 1995). Activation of Rap1 in specific subcellular compartments might be necessary for these cell biological observations.

Recently, a role for Rap1 in controlling integrin-mediated adhesion has been proposed using leukocyte systems. Katagiri et al. showed that LFA-1 (β2 integrin)-mediated adhesion to

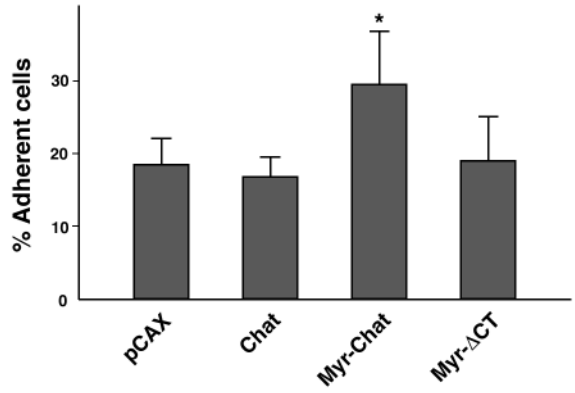

Fig. 10. Myr-Chat expression enhances the cell adhesion on fibronectin. 293T cells were transfected with a control pCAX or indicated Chat variant plasmid together with pCAX-EGFP. Cell adhesion on 96-well culture plates coated with 0.2 μg/ml fibronectin was measured as described in the Materials and Methods. Mean values obtained from the average of six independent wells are shown with s.d. The asterisk indicates the value with a statistically significant increase (P<0.01) over control cells determined by Student's *t*-test. Control and Chat-transfected cells adhered to the fibronectin matrix to a similar extent. Myr-Chat-expressing cells showed a 1.5 times increase of adhesion compared with control cells. Expression of Myr-ΔCT did not enhance the adhesion.

ICAM1 was regulated by Rap1 in BA/F3 B cells expressing LFA-1 (Katagiri et al., 2000). Reedquist et al. described the requirement of Rap1 for CD31-mediated activation of VLA-4 (β1 integrin) and LFA-1 in Jurkat T cells (Reedquist et al., 2000). Caron et al. showed that phagocytosis in macrophages, which is mediated by αMβ2 complement receptor 3, is Rap1 dependent (Caron et al., 2000). In the case of adherent cells, C3G-deficient mouse embryonic fibroblasts showed impaired cell attachment and spreading phenotypes resulted from the inefficient Rap1 activation (Ohba et al., 2001). These lines of evidence indicate that Rap1 controls integrin function. Taken together with our observation that Myr-Chat overexpression modulated cell adhesion, a novel function of the Chat-Cas signaling pathway in regulating integrin adhesion via Rap1 activity is suggested.

The only known tyrosine phosphorylated protein that binds to the Chat SH2 domain is activated Eph receptors (Dodelet et al., 1999). Activation of Eph receptors by ephrin ligands transmits repulsive cell-cell interaction signals, which play pivotal roles in controlling cell migration, axon guidance and compartmental boundary formation during the embryogenesis (reviewed in Holder and Klein, 1999; Schmucker and Zipursky, 2001). Several studies have demonstrated that the ephrin-Eph system controls the adhesion property of integrins (Huynh-Do et al., 1999; Miao et al., 2000), although the mechanism is not known. It is an intriguing possibility that Chat is involved in this regulation of cell adhesion downstream of the ephrin-Eph system.

In the present study, we provided evidence that Chat, an adaptor protein for Cas family proteins, indirectly regulates the activity of Rap1. Furthermore, we proposed a potential regulatory system for cell adhesion by a Chat-Cas signaling pathway through controlling Rap1 activity. This pathway may be involved in the modulation of cell adhesion mediated by certain receptor tyrosine kinases, such as Eph receptors, and may play a role in the development of the multicellular organisms.

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