Chat, a Cas/HEF1-associated Adaptor Protein That Integrates Multiple Signaling Pathways*

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Cas (Crk-associated substrate) and HEF1 (human enhancer of filaments) are related adaptor proteins that function in integrin-mediated cell adhesion and antigen receptor signaling pathways. We report here a molecular cloning of Chat (Cas/HEF1-associated signal transducer) that associates with Cas and HEF1. Chat is a 78-kDa signaling molecule with an N-terminal SH2 domain and is expressed in a wide range of tissues. In hematopoietic cells, a 115-kDa isoform of Chat (Chat-H) was specifically expressed. Chat is associated with Cas in brain, and Chat-H is associated with HEF1 in splenocytes. Deletion analyses revealed that Chat and Cas are associated with each other by their C-terminal domains. Treatment of PC12 cells with epidermal growth factor or nerve growth factor increased the phosphorylation level of Chat. This increase was suppressed by an inhibitor of mitogen-activated protein (MAP) kinase kinase, PD98059, suggesting the phosphorylation of Chat by MAP kinase. In Chat-overexpressed COS7 cells, the activity of c-Jun N-terminal kinase was up-regulated. After the epidermal growth factor stimulation, Chat and Cas were colocalized with actin filaments at ruffling membranes. These findings suggest that Chat transduces signals of tyrosine kinases and MAP kinase to Cas signaling pathway.

Cell adhesion to extracellular matrix (ECM) concerns various biological events, such as cell growth, survival, differentiation, and migration, which is mediated by integrin receptors in a wide range of cell types (reviewed in Refs. 1 and 2). Integrin family proteins consist of heterodimeric transmembrane proteins and have an ECM-binding extracellular domain and a cytoplasmic domain associated with actin cytoskeleton. At the cytoplasmic surface of integrin-ECM adhesion site, subsets of signaling molecules are recruited and form multi-molecular signaling complex.

Tyrosine kinases play a key role in both the integrin-mediated signaling and the regulation of cell adhesion (reviewed in Refs. 3–5). Several lines of evidence suggest that the stimulation of receptor-type tyrosine kinases and the activation of integrin show a synergistic effect on their downstream events (Refs. 6–8 and reviewed in Ref. 9). Integrin-ECM interaction primarily activates focal adhesion kinase (FAK) (10) and Src (4). This activation in turn triggers tyrosine phosphorylation of several signaling molecules and structural proteins including Cas (11, 12), Shc (13), paxillin (14), cortactin (12), and tensin (15), which further activate downstream signals and promote membrane-cytoskeletal reorganizations.

Protein modules, such as Src homology 2 (SH2) and SH3 domains that respectively bind to tyrosine phosphorylated proteins (16) and proline-rich motifs (17), function in protein-protein interaction in these signaling pathways. Several signaling molecules are constituted of only these modules and their binding motifs, and are termed “adaptor proteins” (reviewed in Refs. 18 and 19).

Cas, originally identified as a major tyrosine phosphorylated protein in v-Crk- or v-Src-transformed fibroblasts, belongs to this adaptor protein family (20). Cas contains, from the N terminus, SH3 domain, substrate domain with multiple SH2 binding sites, and SH2 and SH3 domain-binding motifs for Src family kinases (21). FAK is a binding partner of the SH3 domain (22), and when cells attach to ECM, stimulates Src-mediated tyrosine-phosphorylation of Cas through its interaction with Src (23). Protein-tyrosine phosphatases PTP-1B (24) and PTP-PEST (25) also bind to the same domain and suppress the Cas function through the dephosphorylation of Cas.

Integrin-mediated activation of FAK-Cas signaling pathway elicits the activation of c-Jun N-terminal kinase (JNK), which is necessary for proper cell cycle progression (26). In this pathway, tyrosine-phosphorylated residues in substrate domain of Cas serve as multiple binding sites for another adaptor protein, Crk (27). Crk recruits there its downstream effector, DOCK180 (28, 29), which activates Rac1 and causes subsequent activation of JNK. DOCK180-Rac1 pathway also affects the cytoskeletal reorganization and cell spreading (29). Interestingly, overexpression of Cas promotes the membrane ruffling and cell migration on ECM by a Rac1-dependent manner (30), suggesting a similar signaling cascade. Furthermore, several growth factors stimulate Cas-dependent cell migration (30), which suggests that receptor tyrosine kinases transmit signals to Cas.

HEF1/Cas-L (31, 32) and Efs/Sin (33, 34) are members of the Cas family with common structural features. HEF1 is preferentially expressed in lymphocytes (32) and functions in β1 integrin-mediated signaling and antigen receptor signaling pathways (35, 36). The physiological function of Efs/Sin remains elusive. The most C-terminal regions of these proteins including Cas show significantly high sequence similarity to one another; however, the function of these regions has not been characterized yet.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB030442.

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‡ The abbreviations used are: ECM, extracellular matrix; FAK, focal adhesion kinase; SH2 and SH3, Src homology 2 and 3; EGF, epidermal growth factor; NGF, nerve growth factor; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; GST, glutathione S-transferase; HA, hemagglutinin.
In this study, we have isolated a gene encoding an SH2 domain-containing adaptor protein, Chat (Cas/HEF1-associated signal transducer), and identified its hematopoietic cell-specific isoform, Chat-H. Chat and Chat-H bound, respectively, to the C-terminal domains of Cas and HEF1. Furthermore, Chat was phosphorylated by mitogen-activated protein (MAP) kinase after growth factor stimulation, and overexpression of Chat caused JNK activation. We also demonstrated that Chat and Cas were colocalized at ruffling membranes. These results suggest that the Chat-Cas complex regulates the JNK signaling pathway and membrane ruffling at the downstream regions of tyrosine kinases and MAP kinase.

**EXPERIMENTAL PROCEDURES**

**Materials and Cells**—In the process of an immunoscreening of a mouse lung cDNA library constructed in agt11 using a monoclonal antibody BA5/18, the initial clone was isolated by a cross-reaction of the antibody. 2 Using this clone as a probe, full-length mouse cDNA encoding Chat was obtained. After subcloning the insert into pBluescript SK(−) vector (Stratagene), nucleotide sequencing was performed.

Rabbit anti-Chat polyclonal antibodies (anti-Chat SH, amino acid residues 57–167; anti-Chat CT, residues 308–702; anti-Chat CP, residues 57–167) were raised against the SH2 domain and the C-terminal half of Chat, respectively, in E. coli, and a hemocyanin-conjugated synthetic oligopeptide, respectively. Anti-phil34 (anti-Cas/HEF1), recognizing both Cas and HEF1, was purchased from Transduction Laboratories. Anti-phosphotyrosine 4G10 was obtained from Upstate Biotechnology. Anti-FLAG M2 (Sigma) and Anti-HA 3F10 (Roche Molecular Biochemicals) were used for epitope-tagged proteins. Anti-phosphotyrosine 4G10 was obtained from Upstate Biotechnology. Anti-FLAG M2 (Sigma) and Anti-HA 3F10 (Roche Molecular Biochemicals) were used for epitope-tagged proteins.

**PC12 cells** were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 5% horse serum. COS7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

For growth factor stimulation, PC12 cells were incubated with 50 ng/ml epidermal growth factor (EGF; Takara) or 50 ng/ml nerve growth factor (NGF; Upstate Biotechnology) for 3 min. For PD98059 (Research Biochemicals International) treatment, cells were preincubated with 50 μm PD98059 for 30 min before growth factor stimulation. COS7 cells were serum-starved for 16 h in Dulbecco’s modified Eagle’s medium supplemented with 0.5% bovine serum albumin before EGF stimulation.

**Isolation of Hematopoietic Cells**—Mouse lymphoid tissues dissected in RPMI 1640 medium supplemented with 5% fetal bovine serum were passed through a 70-μm nylon mesh. The suspended cells were collected by centrifugation at 300 × g for 10 min. For splenocyte isolation, cells were then resuspended in Tris-NH4Cl solution (0.15 M NH4Cl, 0.01 M Tris-HCl, pH 7.2) to burst red blood cells. After 2 min at room temperature, the suspension was diluted 10-fold with RPMI 1640 medium and collected by centrifugation at 300 × g for 10 min.

**Immunoprecipitation and Immunoblotting**—Mouse tissues were homogenized using a Dounce homogenizer in approximately 20 volumes of ice-cold IP buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM Na2VO3, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A). After centrifugation at 100,000 × g for 30 min, the supernatants were collected as the tissue lysates. To prepare the lysates from isolated lymphoid cells or cultured cells, cells were lysed with IP buffer and gently rotated for 30 min at 4 °C. The samples were centrifuged at 15,000 × g for 20 min, and supernatants were saved. Appropriate antibodies and protein A-Sepharose (Amersham Pharmacia Biotech) were added to the lysates, and the mixtures were rotated for 3 h at 4 °C. Immunoprecipitates were washed five times with IP buffer and then eluted by boiling in the sample buffer for SDS-polyacrylamide gel electrophoresis for 10 min. Alkaline phosphatase treatment of immunoprecipitates was performed as described (37).

For immunoblotting, proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto a nitrocellulose membrane (Protran, 0.22-μm pore size; Schleicher & Schuell). The membrane was incubated with first antibodies, followed by peroxidase-conjugated appropriate secondary antibodies (Amersham Pharmacia Biotech). Immunoreactive signals were detected with Western blot chemiluminescence reagent (NEN Life Science Products).

**Construction of Expression Plasmids and Transfection**—To determine the binding sites for Chat-Cas interaction, expression plasmids for Chat, Cas, and their deletion mutants were constructed. N-terminally FLAG-tagged full-length Chat and a deletion series of Chat (ChatΔ12–705, ChatΔ359–735, ChatΔ359–492, and ChatΔ1–41) were constructed in pCI-neo vector (Promega), after digestion with an oligonucleotide containing the FLAG sequence. Similarly, full-length Cas and its deleted forms (CasΔ325–714, CasΔ511–714, and CasΔ711–714) were constructed in N-terminal HA-tagging vector pCMV-HA. His-tagged full-length Chat (His-Chat) was constructed by adding six histidine codons upstream the initiation codon of Chat and expressed by using pCAX, a derivative of pCMV-HA (38). In JNK assays and immunofluorescence analyses, pCAX was used for the expression of N-terminally FLAG-tagged full-length Chat (pCAX-Chat), and pSSR-Cas (21) was used for the full-length Cas.

For transient expression, transfections were performed using LipofectAMINE plus reagent (Life Tech.) according to the manufacturer’s instructions.

**In Vitro Binding Assay**—His-tagged Chat was expressed in COS7 cells by transient transfection with pCAX-His-Chat. After 24 h, cells were homogenized in His-binding buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM imidazole, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol), and clarified by centrifugation at 15,000 × g for 20 min. His-Bind Resin (Novagen) was mixed with the lysate for 1 h at 4 °C, followed by packaging into a column. The column was washed with His-binding buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM imidazole, 10% glycerol) and eluted with His-elution buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 100 mM imidazole, 10% glycerol).

To express glutathione S-transferase (GST) fusion protein of Chat association domain of Cas (GST-CasΔ711–724) in E. coli, pGEX-4T-1 vector (Amersham Pharmacia Biotech) was used. GST or GST-CasΔ711–724 produced in E. coli was bound to glutathione-Sepharose (Amersham Pharmacia Biotech) in Binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl2, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol). His-Chat produced in COS7 cells was diluted ten-fold with Binding buffer and incubated with GST-bound or GST-CasΔ711–724-bound glutathione-Sepharose for 3 h at 4 °C. After washing five times, the adsorbed materials were subjected to immunoblot with anti-GST Chat CP.

**JNK Assay**—COS7 cells grown on 60-mm dishes were transfected with 1.25 μg each of indicated plasmids as described above. Total amount of transfected DNA was adjusted to 2.5 μg/60-mm dish with control vector pCAX. After 24 h, the cells were serum-starved for 24 h at 37 °C. Preparation of cell lysates and solid phase JNK assay were carried out according to Hibi et al. (39). Briefly, endogenous JNK was pulled down by GST-c-Jun (39). In JNK assays and immunofluorescence analyses, JNK was subjected to kinase reaction in the presence of [γ-32P]ATP. Incorporated radioactivity was quantitated by measuring the signal intensity of the autoradiogram of the samples on SDS-polyacrylamide gel electrophoresis.

**Immunofluorescence Microscopy**—For indirect immunofluorescence microscopy, cells were cultured on cover glasses and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. After permeabilization with 0.2% Triton X-100 in PBS for 15 min, cells were soaked in a blocking solution (PBS containing 1% bovine serum albumin) for 30 min. The specimens were incubated with first antibodies for 60 min in a moist chamber, washed three times with the blocking solution, and then incubated for 60 min with the second antibody, Cy3-labeled goat anti-rabbit IgG, Cy2-labeled goat anti-mouse IgG (Amersham Pharmacia Biotech). To visualize actin filaments, Texas Red-conjugated phalloidin (Molecular Probes) was used. Samples were then washed with PBS for three times, mounted in Perma Fluor (Immuno), and examined using a fluorescence microscope, Zeiss Axioskop II fluorescence microscope (Carl Zeiss).

**RESULTS**

**cDNA Cloning and Expression Profile of Chat**—In the process to identify an antigen that localizes at cell-cell adherence junctions by immunoscreening of an expression library, we isolated a cDNA clone encoding a novel adaptor protein by a cross-reaction of the antibody. This protein had an N-terminal SH2 domain, and its calculated molecular mass was 78 kDa (Fig. 1, A and B). The central portion of this protein was rich in serine, threonine, and proline residues, and this portion contained four consensus sites for MAP kinase phosphorylation (Pro-X-Ser/Thr-Pro) shown by double underlines in Fig. 1A. As described below, this protein was associated with Cas family members 2A. Sakakibara, unpublished data.
Adaptor proteins, Cas and HEF1, through its C-terminal domain (Fig. 1B). Considering these properties, we named this protein Chat.

As the first step, we examined the expression of Chat in various tissues of mouse. Immunoblot analysis using anti-Chat CP polyclonal antibody showed a relatively ubiquitous expression pattern of Chat at the molecular mass of 78 kDa (Fig. 1C, arrow). A 115-kDa isoform of Chat was specifically expressed in hematopoietic cells from spleen, lymph node, and thymus (Fig. 1B, arrowhead). Although the detailed molecular nature of this 115-kDa protein remained to be clarified, we tentatively named the isoform Chat-H, meaning the hematopoietic cell-specific Chat. Same results were obtained with other anti-Chat polyclonal antibodies that recognized distinct epitopes (anti-Chat SH and anti-Chat CT; data not shown).

Identification of Cas and HEF1 as Chat-associated Partners—To seek for a clue to Chat function, we tried to identify a tyrosine phosphorylated protein that formed a complex with Chat, because Chat had one SH2 domain at its N terminus. Chat was immunoprecipitated with anti-Chat-CT from lysates of mouse brain and splenocytes, and immunoprecipitates were subjected to immunoblot analyses (Fig. 2). Anti-Chat-immunoblot confirmed the results in the previous section that brain and lymphoid cells expressed only Chat and Chat-H, respectively (Fig. 2, top panel). Tyrosine-phosphorylated proteins with apparent molecular masses of 130 kDa (in brain) and 115 kDa (in spleen) were detected as major Chat-associated molecules (Fig. 2, middle panel).

We then applied antibodies against various known tyrosine-phosphorylated proteins, the molecular mass of which was between 115 and 130 kDa. An anti-Cas antibody that recognized Cas and HEF1 reacted with both Chat-associated proteins (Fig. 2, bottom panel). This result revealed that the 130-kDa protein and the 115-kDa protein were Cas and HEF1, respectively.

Determination of Binding Sites of Chat and Cas for Chat-Cas Interaction—The essential region of Chat for the association of Cas was determined by co-immunoprecipitation assay using various deletion mutants of Chat and full-length Cas coexpressed in COS7 cells (Fig. 3A). As shown in Fig. 3B, full-length Chat, Chat346-702 and Chat345-702 associated with Cas to a similar extent, indicating that the C-terminal half-region of Chat (Chat345-702) was fully capable of the Chat-Cas interaction. However, the deletion of 146 amino acid residues from the middle (Chat346-492) or 78 residues from the C terminus (Chat345-619) completely disrupted the binding activity. The expression level of these proteins and Cas were similar among these samples (Fig. 3B). These results indicate that the C-
domain of Cas was determined using various HA-tagged Cas constructs (Fig. 4B). To identify the binding site on Cas for the association with Chat, we performed co-immunoprecipitation assays using a series of deletion mutants of Cas and Chat (top panel). The expression levels of Chat and its derivatives (middle panel), and Cas (bottom panel) were analyzed by immunoblots with anti-FLAG and anti-Cas/HEF1 by using total lysates of the same transfectants.

Terminal half-region of Chat but not the SH2 domain is required for the association with Cas, and we named the C-terminal region “Cas association domain” (Fig. 3A).

Cas is known to form a complex with diverse signaling molecules through its SH3, substrate, and Src binding domains (20–22, 24, 25). To identify the binding site on Cas for the association with Chat, we then performed co-immunoprecipitation assay using a series of deletion mutants of Cas and full-length Chat (Fig. 4A). N-terminally deleted mutants (Cas425–874 and Cas671–874) still fully retained the association capacity with Chat (Fig. 4B), demonstrating that the C-terminal 204 residues are sufficient for the association. By the deletion of C-terminal 93 residues, however, Cas completely lost the association activity (Cas1–781, in Fig. 4B), suggesting that Chat-Cas interaction is direct.

Because Chat had four potential MAP kinase phosphorylation sites (Fig. 1A), we examined the effect of an inhibitor of MAP kinase and/or ERK2 kinase, PD98059, on Chat phosphorylation induced by EGF. PD98059 treatment clearly suppressed the upward shift of Chat to a similar extent (top panel). However, Chat in these cells was not recognized by an anti-phosphotyrosine antibody, indicating that Chat was not tyrosine phosphorylated under the condition (middle panel). When anti-Chat immunoprecipitates were treated with calf intestine alkaline phosphatase, the mobility of Chat from both control and EGF-treated cells increased to a same level (top panel). This result indicated that Chat was phosphorylated to some extent in the control cells and that the phosphorylation level further increased by EGF treatment. Because Chat before the phosphatase treatment was detected as a significantly broad band, multiple sites might be phosphorylated. Even after the phosphatase treatment, Cas still bound to Chat, which suggested that the Chat-Cas interaction was phosphorylation-independent (bottom panel).

Activation of JNK by Chat Overexpression—Recent studies reported that JNK is up-regulated when a member of Cas signaling pathway is overexpressed (29, 40). To assess the possibility that Chat is also involved in this signaling pathway, we measured JNK activity of COS7 cells overexpressing Chat (Fig. 6). In Chat-overexpressing cells, JNK activity was 1.7-fold higher than in the control cells (Fig. 6B). This increase was similar to that induced by Cas (1.9-fold (Fig. 6B)) or the data in Ref. 40) or Crk (40). Coexpression of Chat and Cas activated JNK to a similar level (2.0-fold (Fig. 6B)). The amount of JNK did not vary significantly among the samples (data not shown). These results suggest that Chat is also involved in the Cas-mediated JNK activation pathway.
A Novel Cas- and HEF1-associated Adaptor, Chat

**FIG. 5.** Chat is phosphorylated after EGF or NGF stimulation in PC12 cells. **A**, PC12 cells were stimulated with 50 ng/ml EGF (+EGF) or 50 ng/ml NGF (+NGF) for 3 min at 37 °C or left untreated (Control). Anti-Chat CT immunoprecipitates were subjected to immunoblot with anti-Chat CP (top panel), anti-phosphotyrosine (middle panel), or anti-Cas/HEF1 (bottom panel). In the right-hand panels, the immunoprecipitates were incubated in the presence (+AP) or absence (−AP) of alkaline phosphatase with (+PI) or without phosphatase inhibitors prior to immunoblot. **B**, PC12 cells were treated with 50 μM PD98059 (+PD98059) or a control solvent (+DMSO) for 30 min at 37 °C, followed by stimulation with 50 ng/ml EGF (+EGF) for 3 min. Anti-Chat CP immunoblot was carried out as in A.

**FIG. 6.** Effect of Chat and Cas overexpression on JNK activation. COS7 cells were transfected in duplicate with pCAX (Control), pCAX-Chat (Chat), pSSRαCas (Cas), or a mixture of pCAX-Chat and pSSRαCas (Chat + Cas), 2 days after transfection, endogenous JNK activity was determined according to Hibi et al. (39). A, GST-c-Jun-ΔNp1 precipitated JNK activity was visualized by autoradiography of 32P-labeled GST-c-Jun1–79 on SDS-polyacrylamide gel electrophoresis. Duplicate samples (#1 and #2) are shown. B, JNK activity was quantified from digitized image of the autoradiogram by using National Institutes of Health IMAGE analysis program. The values are illustrated relatively taking the value of control cells as 1. Each value (an average of the duplicate samples) represents the mean ± S.E. of three independent experiments. Each transfectants showed statistically significant increase in relative activity over control cells (Chat, p < 0.01; Cas, p < 0.005; and Chat + Cas, p < 0.001) as determined by Student’s test.

Colocalization of Chat and Cas in Living Cells—To further confirm the association of Chat with Cas, we performed immunofluorescence analyses of COS7 cells expressing Chat together with staining of actin cytoskeletal structures (Fig. 7). In Chat-transfected COS7 cells, Chat was predominantly detected throughout the cytoplasm (Fig. 7A). Rapid translocation of Chat to ruffling membranes was observed after stimulation of membrane ruffling by EGF (Fig. 7, C and D) or insulin (data not shown).

Chat and Cas were then expressed simultaneously, and their cellular distributions were studied. In unstimulated cells, both Chat (Fig. 7E) and Cas (Fig. 7F) were mainly localized in the cytoplasm. When cells were EGF-stimulated, intense staining of Chat (Fig. 7G) and Cas (Fig. 7H) at ruffling membranes was observed, and their patterns were very similar to each other. Taken together with the results that Chat and Cas formed a complex in cell lysates, the association of Chat and Cas in living cells was strongly suggested.

**DISCUSSION**

This study demonstrates that Chat, an adaptor protein having an SH2 domain, interacts with the C-terminal domain of Cas family adaptor proteins. Chat was phosphorylated by MAP kinase after the stimulation of tyrosine kinase receptors in PC12 cells. In Chat-overexpressing COS7 cells, the JNK activity was up-regulated. Chat showed colocalization with Cas at ruffling membranes. These results suggest that Chat integrates signals from tyrosine kinases and MAP kinase, controlling cell growth and cytoskeletal reorganization through the Cas signaling pathway.

Very recently, Lu et al. (41) reported a molecular cloning of NSP family proteins, NSP1, NSP2, and NSP3, and it emerged that NSP3 is the human ortholog of mouse Chat (90% identity between NSP3 and Chat amino acid sequences; 35–40% identity between Chat and NSP1 or NSP2). BCAR3 that confers antiestrogen resistance to a mammary cell line (42) is identical to NSP2. Ascidian HrSH2 may also be a member of this family (43). As described here Chat/NSP3 binds to Cas. Lu et al. (41) described the Cas-NSP1 interaction in the same study, although they speculated that the association was mediated by Cas SH3 domain. Furthermore, NSP1 undergoes tyrosine phosphorylation and Chat/NSP3 becomes Ser/Thr phosphorylated upon growth factor stimulation. Therefore, these family proteins may participate in novel signaling pathways to Cas.

Tissue immunoblot analyses showed that Chat was expressed ubiquitously except hematopoietic cells, wherein only a hematopoietic cell-specific isoform, Chat-H, was expressed (Fig. 1C). Chat and Chat-H respectively associates with Cas and HEF1 (Fig. 2). HEF1 is tyrosine phosphorylated when B or T cell antigen receptor is stimulated (32, 35, 36). Chat-H was recovered in anti-phosphotyrosine immunoprecipitates when B cell antigen receptor was cross-linked,2 consistent with the
By using various deletion mutants of Chat and Cas, we demonstrated that C-terminal half of Chat binds to C-terminal 204 amino acids of Cas (Figs. 3 and 4). Although the binding of Chat-H to HEF1 was not fully characterized yet, it was highly likely that complex formation between Chat-H and HEF1 was mediated by their C-terminal domains, because (a) our preliminary characterization of Chat-H revealed that Chat and Chat-H share same C-terminal sequence and (b) the C-terminal region of human HEF1 shows relatively high sequence similarity to Chat association domain of rat Cas (56% identical, 73% conservative among 204 residues). Whether Chat or Chat-H interacts with the third member of Cas family protein, Efs/Sin, remains to be clarified. Similarity between Efs/Sin and Cas (42% identical, 57% conservative among C-terminal 147 residues) is lower both in length and extent compared with that between Cas and HEF1.

Cas family proteins participate in multiple biological events including integrin-mediated cell-matrix adhesion (11, 44), JNK activation (26, 40), cell migration (30), B and T cell antigen receptor signaling (32, 35, 36), and Src-induced transformation (44). Recently, the molecular basis for these events has been intensely investigated. In integrin-mediated cell adhesion, Cas forms a multivalent scaffold complex at focal adhesion sites. This complex includes at least FAK, Src, and Crk as direct binding partners of SH3, Src binding, and substrate domains of Cas, respectively (20–22). The C-terminal regions of Cas family proteins are highly conserved in their primary structures; however, the function of these regions has not been characterized, except that C-terminal portion of HEF1, when expressed in budding yeast, causes the filamentous cell morphology (31). Our result, that Chat binds to the C-terminal domain of Cas (Fig. 4C) and probably to HEF1, reveals a novel function of these domains.
We showed that the phosphorylation level of Chat was elevated when PC12 cells were stimulated by EGF or NGF and suggested MAP kinase as a major kinase responsible for this increase (Fig. 5). The result coincided with the molecular feature of Chat having four MAP kinase phosphorylation sites (Pro-X-Ser/Thr-Pro) in the central region (Fig. 1A). Human NSP3 has five MAP kinase sites in the corresponding region. Interestingly, NSP1 does not have any MAP kinase site in the molecule. It is intriguing to clarify the physiological function of MAP kinase phosphorylation of Chat. Although endogenous Chat in PC12 cells did not undergo tyrosine phosphorylation by EGF (Fig. 5), when overexpressed in COS7 cells, Chat was tyrosine phosphorylated during the stimulation with the same ligand (data not shown). Lu et al. (41) also reported that NSP1 was tyrosine phosphorylated by EGF and that NSP1 bound to activated EGF receptor in a similar overexpression experiment.

Recently, the physiological significance of FAK-Cas signaling pathway in integrin-mediated JNK activation and cell cycle progression was emphasized (26), and several reports demonstrated that overexpression of Cas, or component of Cas signaling pathway, Crk, DOCK180 or NSP1, activates JNK (29, 40, 41). Recently, the physiological significance of FAK-Cas signaling pathway in integrin-mediated JNK activation and cell cycle progression was emphasized (26), and several reports demonstrated that overexpression of Cas, or component of Cas signaling pathway, Crk, DOCK180 or NSP1, activates JNK (29, 40, 41). It is intriguing to clarify the physiological function of MAP kinase phosphorylation of Chat. Although endogenous Chat in PC12 cells did not undergo tyrosine phosphorylation by EGF (Fig. 5), when overexpressed in COS7 cells, Chat was tyrosine phosphorylated during the stimulation with the same ligand (data not shown). Lu et al. (41) also reported that NSP1 was tyrosine phosphorylated by EGF and that NSP1 bound to activated EGF receptor in a similar overexpression experiment.

The recently discovered adaptors Chat and HEF1 were also involved in various signal transduction pathways. The initial phase of this project was carried out in Dr. Tsukita’s laboratory. We thank S. Tsukita for the permission to use the cDNA clone encoding Chat and T. Nakamoto and H. Hirai for providing the Cas expression construct. We also thank S. Nakamura, T. Katagiri, Y. Ohta, Y. Imai, and M. Matsuda for valuable comments and technical advice.

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