Transmembrane Phosphoprotein Cbp Positively Regulates the Activity of the Carboxyl-terminal Src Kinase, Csk*

Received for publication, May 15, 2000, and in revised form, July 27, 2000
Published, JBC Papers in Press, July 28, 2000, DOI 10.1074/jbc.C000326200

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Csk (carboxyl-terminal Src kinase) is a cytoplasmic tyrosine kinase that phosphorylates a critical tyrosine residue in each of the Src family kinases (SFKs) to inhibit their activities. Recently, we identified a transmembrane protein, Cbp (Csk-binding protein), that, when phosphorylated, can recruit Csk to the membrane where the SFKs are located. The Cbp-mediated relocation of Csk to the membrane may play a role in turning off the signaling events initiated by SFKs. To further characterize the Csk-Cbp interaction, we have generated a reconstituted system using soluble, highly purified proteins. Csk and phosphorylated Cbp were co-purified as a large protein complex consisting of at least four Csk-Cbp units. The addition of the phosphorylated, but not nonphosphorylated, Cbp to an in vitro assay stimulated Csk activity toward Src. Csk was also activated by a phosphopeptide containing the tyrosine in Cbp that binds to Csk (Tyr-314). Kinetic analysis revealed that Cbp or the phosphopeptide induced up to a 6-fold reduction in the \( K_m \) for Src, indicating that the Csk-Cbp complex has a greater affinity for Src than free Csk. These findings suggest that Cbp is involved in the regulation of SFKs not only by relocating Csk to the membrane but also by directly activating Csk.

The Src family kinases (SFKs) are nonreceptor protein tyrosine kinases (PTKs) that are associated with the inner surface of plasma membrane through their fatty-acylated amino termini (1). SFKs are known to act as molecular switches that regulate a variety of cellular events, including cell growth and division, cell attachment and movement, differentiation, survival, or death (2). SFKs are ordinarily present in an inactive state in which the phosphorylated carboxyl-terminal regulatory tyrosine binds to its own SH2 domain (3). In response to an external stimulus, an SFK is activated through dephosphorylation of the carboxyl-terminal tyrosine or through binding to another protein that displaces the intramolecular interaction. The phosphorylation of the regulatory tyrosine of SFK is known to be catalyzed by another PTK, Csk (4, 5). In contrast, the phosphatases that activate SFKs have not yet been positively identified, although some candidate molecules have been proposed (6, 7). To understand the regulation of SFKs, it is essential to clarify the regulation mechanism controlling the phosphorylation and dephosphorylation of the critical carboxyl-terminal tyrosine.

Csk is a cytoplasmic PTK consisting of an SH3, an SH2, and another protein that displaces the intramolecular interaction. The phosphorylation of the regulatory tyrosine of SFK is catalyzed by another PTK, Csk (4, 5). In contrast, the phosphatases that activate SFKs have not yet been positively identified, although some candidate molecules have been proposed (6, 7). To understand the regulation of SFKs, it is essential to clarify the regulation mechanism controlling the phosphorylation and dephosphorylation of the critical carboxyl-terminal tyrosine.

To test the hypothesis presented above, we searched for phosphoproteins that can bind tightly to the SH2 domain of Csk and identified a transmembrane phosphoprotein, Cbp (Csk-binding protein) (12). Cbp is involved in the membrane localization of Csk as well as in the Csk-mediated inhibition of Src. When phosphorylated on Tyr-314, Cbp can bind to Csk. Within the plasma membrane, Cbp is exclusively localized in the GM1 ganglioside-enriched detergent-insoluble membrane (DIM) domain, which is thought to play an important role in receptor-mediated signaling and where the majority of SFKs are localized (13–16). These findings suggest that Cbp is a novel component of the regulatory mechanism controlling the activity of SFKs. To further evaluate the role of Cbp in the Csk-mediated regulation of SFKs, we examined the effect of Cbp binding on the kinase activity of Csk. The binding of phosphorylated Cbp or a phosphopeptide containing Tyr-314 could substantially elevate the affinity of Csk for Src.

EXPERIMENTAL PROCEDURES

Protein Expression—Rat Csk cDNA was inserted into pBlueBac4.5 vector and a recombinant baculovirus was generated according to the manufacturer’s instruction manual (Invitrogen). The cDNAs encoding the cytoplasmic domains of Cbp (Cbp\(\Delta N\), amino acids 53–424) and mouse neuron type c-Src (Src, amino acids 9–539) were inserted into the pFastBac HTa vector to generate His-tagged Cbp\(\Delta N\) and His-tagged Src, respectively. The full-length Src cDNA was inserted into the pFastBac vector. Production of the recombinant viruses and the protein expression in Sf9 cells was carried out according to the instruction manual for Bac-to-Bac baculovirus expression system (Life Technologies, Inc.). Infections were done as follows. To prepare nonphosphorylated Cbp\(\Delta N\), the His-tagged Cbp\(\Delta N\) virus was used alone. To prepare phosphorylated Cbp\(\Delta N\), the His-tagged Cbp\(\Delta N\) virus was co-infected with full-length, untagged, Src virus. To prepare His-tagged Src as a substrate for Csk in vitro kinase assays, His-tagged Src virus was used.

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alone, and the purified Src was treated with p-fluorosulfonylbenzoyl-

Adenosine to destroy kinase activity (17). To prepare the Csk-CbpΔN complex, untagged Csk, His-tagged CbpΔN, and untagged full-length Src viruses were co-infected.

**Purification**—To purify untagged Csk, SF9 cells were lysed in a buffer consisting of 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2% Nonidet P-40, 5 mM β-mercaptoethanol, 0.15 mM NaCl, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysate was centrifuged at 20,000 × g for 30 min, and the 2-fold diluted supernatant was applied onto a Q-Sepharose FF column equilibrated with 50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 5 mM β-mercaptoethanol, 0.01% Nonidet P-40, and 5% glycerol. After washing with the same buffer, a linear gradient of 0.075–0.35 M NaCl was applied. Active fractions were diluted 5-fold and applied onto the SP-Sepharose FF column followed by elution with a linear gradient of 0.05–0.25 M NaCl. The active fractions were concentrated on a HiTrapQ HT column and processed on a Superdex 200 column.

For the phosphorylated form of His-tagged CbpΔN and the complex of Csk and His-tagged CbpΔN, lysates were made from co-infected cells in 20 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 10 mM MgCl2, 1% Nonidet P-40, 5 mM β-mercaptoethanol, 0.15 mM NaCl, 1 mM Na3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. The cleared lysate was applied onto a Q-Sepharose FF column equilibrated with Buffer A consisting of 20 mM Tris-HCl, pH 8.0, 5 mM β-mercaptoethanol, 0.01% Nonidet P-40 and 5% glycerol. After washing, materials were eluted with a linear gradient of 0.15–0.45 M NaCl. Active fractions were directly applied onto a Hi-NTA column (Qiagen) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 0.3 mM NaCl, 20 mM imidazole, 0.01% Nonidet P-40 and 5% glycerol. After washing, materials were eluted with 250 mM imidazole in the same buffer. The concentrated eluate was then separated on a Superdex 200 column equilibrated with Buffer B containing 0.5 mM Na3VO4 and 0.4 mM NaCl. For nonphosphorylated His-tagged CbpΔN and His-tagged Src, purification was carried out as described for phosphorylated CbpΔN except that MgCl2 and Na3VO4 were omitted from the buffers.

**Immunoprecipitation and Pull-down Assays**—The DIM fractions were prepared from neonatal rat brains as described previously (12) and solubilized in ODG buffer consisting of 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1% Nonidet P-40, 5 mM β-mercaptoethanol, 0.25 mM NaCl, 1 mM Na3VO4, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF. The cleared lysate was incubated with antibodies or GST-protein fused to the SH2 domain of Csk (GST-

CskSH2) coupled with protein G-Sepharose or glutathione-Sepharose, respectively. The washed beads were then subjected to immunoblot analysis with anti-phosphotyrosine (pY) and anti-Cbp antibodies. Production of GST-CskSH2 and immunoblot analysis were done as described previously (12). In vitro phosphorylation of Cbp in the DIM fraction was carried out as described (12). The phosphopeptides were synthesized with ACT44omega (Advanced ChemTech) and purified by reverse-phase high pressure liquid chromatography.

**Csk Assay**—Csk or CskΔN complex was incubated for 10 min at 30 °C in a reaction mixture (20 μl) consisting of 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 10 μM [γ-32P]ATP (2 μCi), 0.5 mM Na3VO4, 0.25 mM NaCl, and His-tagged Src in the presence or absence of effectors. The reaction was terminated by the addition of 20 μl of 2× SDS sample buffer and separated by SDS-PAGE. The activity was visualized and quantified using a BAS300 Bioimage Analyzer (Fuji).

**RESULTS AND DISCUSSION**

**Purification of the Csk-Cbp Complex**—To characterize the biochemical features of the Csk-Cbp interaction, we employed a baculovirus expression system. The following recombinant baculoviruses were generated: Cbp lacking the amino-terminal membrane association domain and carrying a His tag (CbpΔN); untagged Csk; intact, untagged Src (as a donor of Cbp phosphorylation); and a truncated Src lacking its amino-terminal membrane association domain and carrying a His tag (His-Src, as a substrate for Csk). The phosphorylated CbpΔN (pCbpΔN) nonphosphorylated CbpΔN (nCbpΔN), His-Src, Csk-CbpΔN complex, and Csk were purified from infected insect cells through sequential column chromatographies as described under “Experimental Procedures” (Fig. 1A). pCbpΔN migrated slowly on the SDS-polyacrylamide gel as an 85-kDa protein, whereas nCbpΔN migrated as a 65-kDa protein. Because the molecular size of the His-tagged CbpΔN predicted from the DNA sequence is about 43 kDa, the composition of amino acids and the phosphorylation states might greatly affect the mobility of the proteins on the gel (12). Co-expression of CbpΔN, Csk, and Src successfully induced an efficient phosphorylation of CbpΔN to generate a substantial amount of the Csk-CbpΔN complex applicable to biochemical analysis. As described previously (12), the interaction was so stable that it was resistant against high concentrations of salt (up to 1 mM NaCl). During purification, the Src protein was completely eliminated from the complex, confirming that there is no close association between Cbp and Src. Upon gel filtration chromatography, the complex was eluted as a large protein complex with a molecular mass of ~440 kDa (Fig. 1B, upper panel). When analyzed for pCbpΔN alone, it gave an apparent molecular mass of ~200 kDa (Fig. 1B, lower panel), suggesting at least that cytoplasmic fraction of Csk is capable of being oligomerized even without Csk association. From these observations, together with the calculated molecular masses of Csk (50 kDa) and His-tagged CbpΔN (43 kDa), it is predicted that the complex consists of at least four sets of Csk-Cbp units. Although further analysis should be undertaken to determine the actual composition of the complex, it seems likely that Csk is concentrated on the...
surface of the oligomerized Cbp. Indeed, gel filtration analysis of the DIM fraction from neonatal rat brain revealed that the native form of Csk-Cbp complex also behaved as a huge protein complex greater than 500 kDa (data not shown). This suggests that the Csk-Cbp complex is oligomerized in vivo as well.

**Competition of Csk-Cbp Interaction with Phosphopeptide**—To further define the condition of the Csk-Cbp interaction, we generated a set of phosphopeptides containing phosphorylation sites of Cbp (Fig. 2A). Mutational analysis had shown that Tyr-314 of Cbp is a critical site for the binding to the SH2 domain of Csk and that the surrounding 4–5 amino acids are sufficient to create the binding site.

**Activation of Csk by Phosphorylated Cbp or Phosphopeptide**—To examine the effect of Cbp binding on the kinase activity of Csk, we generated a set of phosphopeptides containing phosphorylation sites of Cbp (Fig. 2A). Mutational analysis had shown that Tyr-314 of Cbp is a critical site for the binding to the SH2 domain of Csk and that the surrounding 4–5 amino acids are sufficient to create the binding site.

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Csk Activation by Cbp

Cbp activates Csk, we performed a kinetic analysis using the purified Csk-CbpΔN complex. A substrate saturation curve of the complex was compared with that of floating Csk (Fig. 4A). From the Lineweaver-Burk plots (Fig. 4B), the $K_m$ value of the complex for Src was calculated to be $\approx 0.21 \mu M$, which was about 6 times less than that of free Csk (1.27 μM). Binding to Cbp-4 could also reduce the $K_m$ value for Src (0.59 μM). These observations demonstrate that Cbp binding could modulate the conformation of Csk to increase the affinity for protein (Src) or peptide (polyEY) substrates. It was shown previously by mutational analysis that the SH2 and SH3 domains of Csk are important for optimal kinase activity but apparently play no direct or specific role in substrate recognition (19). In this study, however, binding to phosphorylated Cbp or phosphopeptide could modulate the affinity of Csk for the native substrate Src. Structural analysis should help elucidate the molecular basis of these phenomena.

In this study, we show that 1) Csk and the cytoplasmic domain of phosphorylated Cbp formed a stable complex that could be purified to near homogeneity, 2) the Csk-Cbp complex was oligomerized to be a large molecular complex, and 3) the complex formation could enhance the affinity of Csk toward Src. Taken together, these findings predict that Cbp acts as a positive regulator for Csk by recruiting Csk to the membrane where SFKs are present, by concentrating Csk on the oligomerized Cbp, and by directly activating Csk. By these combined mechanisms, the efficient regulation of SFKs may be achieved in vivo. To apply this hypothesis to an in vivo condition, the timing and mechanism of Cbp phosphorylation needs to be investigated. In this context, the roles of potentially responsible kinases, SFKs, and counteracting protein tyrosine phosphatases are now under investigation.

Acknowledgments—We thank T. Hirose for production of recombinant Csk in Sf9 cells, S. Aimoto for peptide synthesis, and J. A. Cooper for critical comments.

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