Genetic Evidence for a Tyrosine Kinase Cascade Preceding the Mitogen-activated Protein Kinase Cascade in Vertebrate G Protein Signaling*

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The signal transduction pathway from heterotrimeric G proteins to the mitogen-activated protein kinase (MAPK) cascade is best understood in the yeast mating pheromone response, in which a serine/threonine protein kinase (STE20) serves as the critical linking component. Little is known in metazoans on how G proteins and the MAPK cascade are coupled. Here we provide genetic and biochemical evidence that a tyrosine kinase cascade bridges G proteins and the MAPK pathway in vertebrate cells. Targeted deletion of tyrosine kinase Csk in avian B lymphoma cells blocks the stimulation of MAPK by Gαs, but not Gαi, coupled receptors. In cells deficient in Bruton’s tyrosine kinase (Btk), Gαi-coupled receptors failed to activate MAPK, while Gαs-coupled receptor-mediated stimulation is unaffected. Taken together with our previous data on tyrosine kinases Lyn and Syk, the Gαi-coupled pathway requires tyrosine kinases Csk, Lyn, and Syk, while the Gαs-coupled pathway requires tyrosine kinases Btk and Syk to feed into the MAPK cascade in these cells. The central role of Syk is further strengthened by data showing that Syk can bind to purified Lyn, Csk, or Btk.

Many neurotransmitters, hormones, chemokines, and sensory stimuli initiate physiological effects through their membrane-bound seven-helix transmembrane receptors that are coupled to heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) (1–3). G proteins transduce signals like an on and off switch. Liganded receptors activate G proteins by catalyzing the exchange of GDP bound to the α subunit (the inactive state) with GTP (the active state), resulting in the dissociation of Gα-GTP from the βγ subunits. Both α and βγ subunits can trigger downstream events (4). Several major intracellular signaling pathways have been shown to be regulated by G protein-coupled receptors. These pathways include the cAMP/protein kinase A pathway, the phosphatidylinositol/calcium/protein kinase C pathway and the mitogen-activated protein kinase (MAPK)1 pathway (1, 5, 6). The coupling mechanisms of G proteins to the cAMP and phosphatidylinositol pathways have been defined (1). Gαs and Gαi proteins directly activate the adenylyl cyclases, thus the cellular level of the second messenger cAMP. Gαs and Gαi proteins directly activate phospholipase Cβ, thus increasing phosphatidylinositol turnover. However, the mechanism by which G protein-coupled receptors activate the MAPK pathway is less clearly defined in vertebrates and is an area of intense research.

The MAPK pathway is found ubiquitously in eukaryotic organisms and is used for regulating cell proliferation, differentiation, and many other diverse biological functions (7–11). This cascade can be activated by a variety of receptors, including G protein-coupled receptors (12–14). The best understood coupling mechanisms to the MAPK pathway are from receptors with intrinsic tyrosine kinase activity in invertebrates and vertebrates (15–17) and from receptors coupled to heterotrimeric G proteins in yeast (11). With receptor tyrosine kinases, extensive and elegant biochemical and genetic work has led to the elucidation of the coupling mechanism (15–17). Following ligand binding and receptor autophosphorylation, the complex of the adapter protein Grb2 and the guanine nucleotide exchange factor Sos, or the Shc (another adapter protein) Grb2-Sos complex, is recruited to tyrosine-phosphorylated receptors. This leads to the prototype Ras/Raf/MEK/MAPK pathway (7–10). In the yeast Saccharomyces cerevisiae mating pheromone response, upon pheromone receptor activation, an inactive heterotrimeric G protein dissociates, freeing the βγ subunits from an inhibitory α subunit. βγ is then able to activate the MAPK cascade through a scaffold protein (STE5), a serine/threonine protein kinase (STE20), and possibly other proteins (11). Our understanding of the route leading from G protein-mediated signals to the MAPK activation in vertebrate cells is much less complete. A number of G protein-coupled receptors have been shown to activate the MAPK cascade. Ras, Raf, protein kinase C, Ca2+, and tyrosine kinase-dependent and -independent pathways have been observed in certain cell types (18–36).

We have begun a molecular genetic analysis of the activation pathway from G proteins to MAPK in vertebrates (30). We have chosen an avian lymphoma cell line DT40 as our model system due to the high efficiency of homologous recombination in these cells (37). Thus it is feasible for genetic manipulation in both generating loss-of-function mutants of relevant molecules by site-specific targeted deletion and in performing gain-of-func-

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* This work was supported by grants from the National Institutes of Health, the National Science Foundation, and the American Heart Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: MAPK, mitogen-activated protein kinase; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein; mAChR, muscarinic acetylcholine receptor; PH, pleckstrin-homology; BCR, B-cell receptor.
tion assay by overexpressing. Using tyrosine kinases Lyn and Syk knock-out cells, we have shown previously that the Gz2- MAPK signaling requires Lyn and Syk, while the Gz4-MAPK signaling needs Syk (30). Here with tyrosine kinases Csk and Btk knock-out cells, we demonstrate that while Csk is required for Gz4-MAPK pathway, Btk is essential for Gz2-MAPK signaling. Furthermore, with epistatic genetic and biochemical analysis in these tyrosine kinase-deficient cells, we have determined the order of action of these tyrosine kinases in the pathways. A surprising positive function for Csk, an otherwise negative regulator of Src-family tyrosine kinases, has been observed. Syk serves as a converging point and plays a central role in both Gz2- and Gz4-coupled signaling pathways. This point has been further strengthened by direct protein-protein binding analysis showing that Syk binds purified Lyn, Csk, or Btk. Therefore, these tyrosine kinases form a cascade that couples G proteins to the MAPK cascade in these avian lymphoma cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—DT40** avian B lymphoma cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 5% chicken serum, 2 mM L-mercaptoethanol, 2 mM glutamine, and 1% penicillin/streptomycin (30). Transient transfections were carried out with 2 μg of plasmid DNA and 5 μl of LipofectAMINE (Life Technologies, Inc.) reagent in six-well culture plates (30).

**Immunoprecipitation and Immunoblot Analysis—DT40** whole cell extracts are prepared as follows: confluent cells are harvested from 10-cm plates, washed twice with cold phosphate-buffered saline, and pellets are resuspended in 0.8 ml of extraction buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.1% SDS, 0.2% Triton X-100). Resuspended pellets are passed five times through a 26-gauge needle, centrifuged at 5000 rpm for 3 min at 4 °C to remove insoluble material, and the supernatant is saved as the whole cell extract.

For immunoprecipitation, 10 μl of protein A-agarose (for polyclonal antibody) or protein G-agarose (for monoclonal antibody) was added to the whole cell lysate to pre-clear (38). Then 5 μl of primary antibody was added and continuously incubated at 4 °C for 30 min. After another 2-h incubation with 30 μl of protein A- or G-agarose beads, the immunocomplex was washed three times with the extraction buffer and three times with wash buffer (10 mM Tris, pH 7.4, 1 mM EDTA). The immunocomplex was then subjected to SDS-PAGE. Immunoblotting for ERK-1, Lyn, Syk, and Btk was done as described (30). Membrane filters were incubated in 1 x Tris-buffered saline, 5% milk for 1 h, then incubated in primary antibody for 2 h at room temperature. Blots were washed three times with Tris-buffered saline/Tween 20 and one time with Tris-buffered saline, then incubated with secondary antibody for 2 h at room temperature. Blots were washed again and signal was detected with ECL (NEN Life Science Products).

**MAPK Assay—Expression of mACHRs in transfected cells was assessed by saturation ligand-binding analysis with intact cells using the muscarinic antagonist [1H]methylyscopolamine (39). Untransfected DT40 cells showed virtually no detectable [1H]methylyscopolamine binding. m1 or m2 mACHR transfected cells had ~40,000 binding sites/cell. After 24 h, transfected cells were starved in serum-free medium for 12 h and whole cell extracts were made after stimulation with carbachol (100 μM) for 5 min (30). ERK-1 immunoprecipitation was done with a monoclonal antibody to ERK-1 (Transduction Laboratory). The MAPK assay was performed as described (30). 10 μg of MBP was used as substrate. Kinase assay buffer was: 30 mM Tris-HCl, pH 8.2, 20 mM MgCl2, 2 mM MnCl2, 10 μM ATP. The mixture was preincubated 3 min before 10 μCi of [γ-32P]ATP was added. After 10 min at 30 °C, samples were separated by 12% SDS-PAGE. Gels were transferred to nitrocellulose membrane filters and exposed for autoradiography. Western blot analysis was done with the anti-ERK-1 monoclonal antibody. Quantitation was performed using a Molecular Dynamics PhosphorImager.

**Tyrosine Kinase Assays—**Whole cell extracts were made after stimulation with carbachol (100 μM for 20 s for m1 mACHR; 1 min for m2 mACHR). Csk immunoprecipitation was done with a monoclonal antibody to Csk (Transduction Laboratory), Lyn immunoprecipitation with a polyclonal antibody to Lyn (40), and Syk immunoprecipitation with a polyclonal antibody to Syk (Upstate Biotechnology, Inc.). Csk kinase assay was done with 10 μg of α-casein as substrate (41). Btk, Lyn, and Syk kinase assays were done with 5 μg of the purified glutathione S-transferase and the cytoplasmic domain of human erythrocyte band 3 fusion protein (GST-CDB3) as substrate. The GST-CDB3 plasmid was constructed by polymerase chain reaction cloning of CDB3 from the plasmid pCD-B/M1 (42). The kinase buffer was: 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 10 μM ATP, 10 μCi of [γ-32P]ATP. After 30 min at 30 °C, samples were separated by 10% SDS-PAGE. Gels were transferred to nitrocellulose membrane filters and exposed for autoradiography. Quantitation was performed using a Molecular Dynamics PhosphorImager.

**Binding Assay—**Whole-cell extracts were dialyzed overnight at 4 °C in phosphate-buffered saline, 0.1% Triton X-100. For binding, 30 μl of nickel-nitriolactic acid agarose beads with bound His-tagged protein (1–2 μg) was added to ~500 μg of whole-cell extract and incubated with gentle mixing for 1 h at 4 °C. The complex is pelleted by spinning at 10,000 rpm for 5 min and washed extensively with phosphate-buffered saline, 0.1% Triton X-100. The washed pellet was resuspended in 2 x sample buffer, boiled 5 min, spun down at 10,000 rpm for 2 min, and loaded onto a 12.5% SDS-PAGE gel. Separated proteins were transferred to nitrocellulose membrane for Western blot analysis.

**RESULTS**

**Csk Is Positively Required for Gz4, but Not Gz2, Signaling to MAPK—**To test whether the COOH-terminal-Src kinase (Csk) plays a role in G protein signaling, we have examined the vertebrate G protein-MAPK pathway in Csk-deficient avian B lymphoma cells (DT40 cells) (43). MAPK was immunoprecipitated with a monoclonal antibody against ERK-1, and its activity was measured by an immune-complex kinase assay using MBP as substrate (30). Western blot analysis with monoclonal antibodies to ERK-1 or ERK-2 revealed that DT40 cells only express ERK-1, not ERK-2 (30). As we reported previously, both Gz4-coupled m1 mACHR and Gz2-coupled m2 mACHR can stimulate (−3–4-fold) the activity of MAPK in DT40 cells after treatment with the agonist carbachol (Fig. 1A). In Csk-deficient DT40 cells (Csk−/− cells), the basal MAPK activity was similar to the wild-type DT40 cells (Fig. 1, B and D). Stimulation of m2 mACHR increased the MAPK activity in Csk−/− cells to a similar level as it did in m2 mACHR-transfected wild-type DT40 cells (Fig. 1B). However, stimulation of m1 mACHR was unable to increase the MAPK activity above basal level, despite similar amounts of mACHRs and MAPK in all these cells (Fig. 1B).

To address whether the failure of stimulation by m1 mACHR was the result of absence of Csk, we transfected wild-type Csk back into Csk−/− cells. As shown in Fig. 1B, Csk expression in Csk−/− cells restored the stimulation of MAPK in response to m1 mAChR. Epidermal growth factor receptor-mediated MAPK activation in Csk−/− cells is not impaired (data not shown). These data suggest that while Csk is required for Gz4-coupled m1 mACHR-mediated activation of MAPK, it is not essential for Gz2-coupled m2 mACHR-mediated activation.

**Btk Is Required for Gz2, but Not Gz4, Signaling to MAPK—**We next examined the role of Bruton’s tyrosine kinase (Btk) in vertebrate G protein-MAPK pathway by studying the activation of MAPK by Gz2- and Gz4-coupled receptors in Btk-deficient cells (Btk−/− cells) (44). The Btk subfamily of nonreceptor tyrosine kinase contains one pleckstrin-homology (PH) domain (45). Defects in Btk are responsible for X chromosome-linked agammaglobulinemia in humans and X chromosome-linked immunodeficiency in mice (for review, see Ref. 46). The basal MAPK activity in Btk−/− cells is similar to the wild-type DT40 cells (Fig. 1, C and D). Stimulation of m1 mACHR increased the MAPK activity in Btk−/− cells to a similar level as it did in m1 mACHR-transfected wild-type DT40 cells (Fig. 1C). However, stimulation of m2 mACHR was unable to increase the MAPK activity above basal level (Fig. 1C). To address whether the failure of stimulation by m2 mACHR was the result of absence of Btk, we transfected wild-type Btk back into Btk−/− cells. As shown in Fig. 1C, reconstitution of Btk restored the stimulation
of MAPK in response to m2 mAChR. Activation of MAPK by epidermal growth factor receptor in Btk<sup>-/-</sup> cells is not affected (data not shown). These data demonstrate that while Btk is required for G<sub>q</sub>-mediated activation of MAPK, it is not essential for G<sub>q</sub>-coupled activation.

Combined with our previous data showing the essential roles of Lyn and Syk in the G<sub>q</sub>-MAPK pathway and of Syk in the G<sub>q</sub>-MAPK pathway (30), it is clear that there are multiple tyrosine kinases involved in both G<sub>q</sub>- and G<sub>i</sub>-MAPK pathways. Therefore, we decided to determine the working order of Csk, Lyn, and Syk in the G<sub>q</sub>-MAPK pathway and of Btk and Syk in the G<sub>i</sub>-MAPK pathway.

Both Csk and Lyn Are Essential for Syk Activation—Csk was originally purified as a kinase that negatively regulates the activity of Src-family tyrosine kinases, including Lyn (47). Thus, it seems puzzling that both Csk and Lyn are positively required for the G<sub>q</sub>-MAPK pathway. To address this paradox, we examined the activation of Csk by G<sub>q</sub>-coupled m1 mAChR in Lyn<sup>-/-</sup> cells and of Lyn in Csk<sup>-/-</sup> cells. Csk kinase activity was measured by an immune-complex kinase assay using α-casein as substrate (41). Both m1 and m2 mAChRs can stimulate kinase activity in wild-type DT40 cells (Fig. 2, A and E). In Lyn<sup>-/-</sup> cells, activation of Csk by m1 mAChR is the same as in wild-type cells (Fig. 2B). In Csk<sup>-/-</sup> cells, Lyn kinase activity can still be increased by m1 mAChR, despite a small increase of basal Lyn activity in Csk<sup>-/-</sup> cells (43) (Fig. 2C). Thus the activation of Csk and Lyn is independent of each other. Although the small increase of basal Lyn activity indicates that Csk could negatively regulate Lyn activity, the positive requirement of Csk for the MAPK activation suggests that in G<sub>q</sub>-MAPK signaling, Csk acts mainly on a target(s) other than Lyn. A similar conclusion has been recently reached from studies on normal lymphocyte differentiation: both Csk- and Src-related kinases are positively required, and Csk acts on targets other than Src-related kinases (48).

We had previously shown that Lyn acts upstream of Syk in the G<sub>q</sub>-MAPK pathway (30). To determine the relationship between Csk and Syk in the G<sub>q</sub>-MAPK pathway, we examined the activation of Syk by m1 mAChR in Csk<sup>-/-</sup> cells and of Csk in Syk<sup>-/-</sup> cells (40). In Csk<sup>-/-</sup> cells, the activation of Syk by m1 mAChR is greatly attenuated (Fig. 2D). In contrast, the activation of Csk in Syk<sup>-/-</sup> cells by m1 mAChR is the same as in wild-type cells (Fig. 2B). Thus Csk functions upstream of Syk. Therefore, both Csk and Lyn are positively required for Syk
Domains—

Both Csk and Btk have distinct structural domains for coupling Gq to MAPK and that the function of the SH2 domain, at least in part, is to recruit Csk to the membrane, which is consistent with the previous report (49, 50). We also tested Btk mutants with dysfunctional PH, SH2, or kinase domains (44). None of these domain mutants could rescue the response to Gβ- or m2 mAChR stimulation (Fig. 4A). These results indicate that all three domains of Btk are essential for Gβ stimulation of MAPK.

**Stimulation of Syk Kinase Activity by Overexpressing Lyn, Csk, and Btk**—The above functional studies with loss-of-function mutants of several tyrosine kinases indicate that these kinases act in a cascade and that Syk sits at a converging point downstream of Lyn, Csk, and Btk. To extend this observation further, we used a gain-of-function assay and examined whether overexpression of Lyn, Csk, or Btk could increase Syk kinase activity. As shown in Fig. 5A, overexpression of Lyn, Csk, or Btk led to increased Syk kinase activity. Therefore, these data are consistent with the genetic requirement of Lyn, Btk, and Csk for Syk activation.

**Syk Binds Purified Lyn, Csk, and Btk**—Although the above genetic data pointed to the possibility of interactions of these tyrosine kinases, to test whether these tyrosine kinases interact directly, we have performed direct binding analysis with purified tyrosine kinases. To simplify the purification of the domain-mutated tyrosine kinases, transfection of CskΔSH3 (SH3 deletion mutant), CskΔSH2 (SH2 deletion mutant), or catalytically inactive mutant CskΔK’ (catalytically inactive mutant CskΔK’) constructs into Csk−/− cells did not restore the stimulation of MAPK by Gq-coupled m1 mAChR. The SH2 and SH3 deletion mutants have no effect on Csk catalytic activity. Addition of the Src membrane-targeting signal to CskΔSH2 (Src-CskΔSH2) partially rescued the response, while addition of the Src membrane-targeting signal to CskΔSH3 (Src-CskΔSH3) did not. None of the PH (btk(mPH)) or SH2 (btk(mSH2)) or kinase-domain (btk(K)) mutants of Btk could reconstitute the response to Gβ-coupled m2 mAChR. The PH and SH2 domain mutants have no effects on the catalytic activity of Btk. In A and B, bottom panels show Western blot analysis of MAPK, Csk and its mutants, Btk and its mutants. Data are representative of three similar experiments.

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kinases, Lyn, Csk, and Btk cDNAs were subcloned into a vector with COOH-terminal hexahistidine tag. Purified Lyn-His$_6$, Csk-His$_6$, and Btk-His$_6$ from *Escherichia coli* have protein kinase activities (detailed kinase characterization will be presented elsewhere). As shown in Fig. 5B, purified Csk, Lyn, and Btk bound to the endogenous Syk from the DT40 whole-cell extracts, while a control His$_6$-tagged protein (NF-AT-His$_6$, a T-cell-specific transcription factor) did not. We were also able to show binding of Lyn to Csk (data not shown). Btk did not bind to either Lyn or Csk (data not shown). These experiments demonstrate that Syk is able to form complexes with Lyn, Csk, and Btk. Thus these tyrosine kinases could form a multimolecular complex, as is the case with the MAPK module (11).

**DISCUSSION**

Our molecular genetic and biochemical analysis of the vertebrate G protein-MAPK pathway places Syk kinase at a critical converging point (Fig. 6A). G$_q$-coupled receptors through Csk and Lyn, and G$_i$-coupled receptors through Btk, activate Syk. Syk, in turn, feeds the signal into the MAPK cascade (Fig. 6A). Although we have drawn the genetic pathway from G protein to MAPK in DT40 cells as a linear pathway, it should be stressed that the relationship among tyrosine kinases could be more complex than the pathway shown, because of the existence of multiple pathways (11). In vertebrate cells, such as DT40 cells, a tyrosine kinase cascade links the G protein to the MAPK cascade. In the case of receptors with tyrosine kinase activity, the signal transmission from the activated tyrosine kinase is accomplished by the recruitment of the small G protein RAS. RAS then switches on a cascade of protein phosphorylation events. Our surprising finding that Csk plays a positive role in the activation of MAPK by G$_q$ signal and in Syk activation in DT40 cells is very intriguing. Csk was originally purified as a kinase that phosphorylates the COOH-terminal tyrosine residues of Src-family tyrosine kinases, including Lyn, thereby negatively regulating their activity (47). Overexpression of Csk decreased T-cell receptor-induced lymphokine production and protein tyrosine phosphorylation in T lymphocytes, although the *in vivo* function of Csk in T-cell receptor signaling is still not clear (49, 50). Mice deficient in Csk have increased activity of Src-family kinases and exhibit impaired formation of neural tube and embryonic lethality (51, 52). However, although Src-family kinases were activated, tumorigenesis was not observed in any tissues. Our data show that Csk is positively required for the G$_q$-MAPK pathway and that overexpressing Csk leads to increased, rather than decreased, Syk kinase activity. Furthermore, overexpressing Csk did not block the stimulation of MAPK by m1 or m2 mAChRs in wild-type DT40 cells. There are several recent reports suggesting that Csk has functions other than suppressing Src-family kinases. Overexpressing Csk in HeLa cells did not significantly suppress Src activity, but still led to dramatic changes in HeLa cell morphology (53). Csk is required for normal development of lymphoid cells. Csk deficiency blocks T- and B-cell differentiation as is the case with Src-family kinase deficiency. Thus Csk could act on targets other than Src-related kinases (48). It has also been shown that Csk could phosphorylate and activate the cell-surface receptor protein-tyrosine phosphatase (54). Therefore, Csk could

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2 K. Bence and X.-Y. Huang, submitted for publication.

3 Y. Wan and X.-Y. Huang, unpublished data.
Could Syk Serve as a Scaffold Protein as Well as a Kinase?—In this report, we have presented data that demonstrate the central role of Syk in both G\(_{\alpha}\) and G\(_{\beta}\) signaling pathways to MAPK. Overexpression of Lyn, Csk, or Btk leads to stimulation of Syk kinase activity. Purified Lyn, Csk, and Btk directly bind to Syk. Coimmunoprecipitation studies have indicated that additional cellular proteins can interact with Syk, including protein kinase C\(\mu\) (55, 56), another Src-family member Lck (57), PLC\(\gamma\)1 and -\(\gamma\)2 (44, 56), the adapter protein SHC (58), as well as the B-cell receptor, the T-cell receptor, and the high-affinity receptor for immunoglobulin E (46, 59). It would appear from these various reports that the composition of Syk-containing signaling complexes is dictated by the nature of the activated receptor and the other signaling molecules involved. It is possible that tyrosine phosphorylation of Syk, like tyrosine kinase FAK, can create specific binding sites for SH2 domain-containing proteins, thereby promoting the formation of intracellular multimeric signaling complexes (60). Thus, Syk could function as a scaffold or adapter protein as well as serve as a kinase. Recently it has been shown that Syk-deficient mice exhibited perinatal lethality (61, 62). It has been suggested that Syk has multiple functions in the mouse such as in maintaining vascular integrity, in wound healing, and in B-cell development (61, 62).

Role of Nonreceptor Tyrosine Kinases in Heterotrimeric G Protein Signaling—Our data demonstrate an essential role for nonreceptor tyrosine kinases in heterotrimeric G protein signaling. Although studies on tyrosine kinases have been very intense over the past decade, regulation of nonreceptor tyrosine kinases by heterotrimeric G proteins has not been studied until recently. Given the broad usage of both the heterotrimeric G protein signaling system and nonreceptor tyrosine kinases in cellular functions, it is very desirable to further our understanding how these two common signaling components communicate to each other. It is not surprising that tyrosine kinases play critical roles in many physiological responses mediated by G protein-coupled receptors. For example, smooth muscle contraction induced by mACHRs, angiotensin II, and vasopressin receptors is blocked by various tyrosine kinase inhibitors (for review, see Ref. 63). G-protein-coupled angiotensin II receptor has been shown to stimulate tyrosine kinases Jak2 and Tyk2 in rat aortic smooth muscle cells leading to the activation of Jak/STAT pathway (64). A variety of G-protein-coupled receptors can stimulate the activity of tyrosine kinase FAK (65). Other documented G protein-coupled receptor-induced events that involve tyrosine kinases include chemotaxis through chemokine receptors, neuronal growth cone collapse by lysophosphatidic acid, cardiovascular hypertrophy/hyperplasia in hypertension, thrombin-induced platelet aggregation, focal adhesion assembly, and stress fiber formation (66–68).

Although the mechanism by which G proteins regulate the activity of adenyl cyclases and phospholipase C-\(\beta\) is well understood, how G proteins activate tyrosine kinases remains unknown. The activation could be direct and indirect. Clearly, elucidation of detailed activation mechanisms of these tyrosine kinases by G proteins requires further biochemical studies. Regardless of the mechanism, G\(_{\alpha}\) and G\(_{\beta}\)-mediated activations and interplays among the tyrosine kinases must be different, since both G\(_{\alpha}\) and G\(_{\beta}\) can stimulate all four tyrosine kinases, but their pathways to MAPK are different. For example, both G\(_{\alpha}\) and G\(_{\beta}\)-coupled receptors are able to stimulate Csk and Btk, but the requirement of Csk and Btk for the G\(_{\alpha}\) and G\(_{\beta}\)-MAPK pathways is different. We do not have a satisfactory answer for how the specificity is achieved, as in the cases of other complicated signal transduction pathways. Full activation of many tyrosine kinases requires phosphorylation of tyrosine residues (either through autophosphorylation or by another tyrosine kinase) (69). It is likely that the tyrosine phosphorylation event could serve as the activation mechanism. In the case of the first tyrosine kinase in a tyrosine kinase cascade, other activation mechanism(s) must exist. Since receptor tyrosine kinases and possibly Raf kinase can be activated by oligomerization (70, 71), it is possible that G proteins translocate tyrosine kinase to the membrane to increase the local concentration leading to the activation of tyrosine kinase. This notion is consistent with our results, indicating that membrane localization of Csk is critical for its function in G protein signaling. It is also possible that removal of the inhibitory mechanism (either an inhibitory factor or an unfavorable conformation) can trigger the activation of these kinases. Unraveling the molecular details of the G protein-tyrosine kinase connection is one of the major challenges for future studies.

Tyrosine Kinase-dependent Activation of the MAPK Pathway by G Protein-coupled Receptors—Several very recent publications have provided strong evidence that, at least in some vertebrate cell types, activation of the MAPK pathway by G protein-coupled receptors is tyrosine kinase-dependent (30–34). The identity of the involved tyrosine kinases is beginning to be defined. We have used a genetic approach to reveal the roles of tyrosine kinases Lyn, Csk, Btk, and Syk in G protein-MAPK pathways in avian lymphoma cells. A dominant-negative mutant of another tyrosine kinase Pyk2 has been shown to interfere the signaling from G\(_{\alpha}\)-coupled bradykinin receptor and G\(_{\beta}\)-coupled lysophosphatidic acid receptor to MAPK in PC12 cells (Ref. 34; but not in rat liver epithelial cell lines (72)). Src-family tyrosine kinases Fyn and Src have also recently been implicated in G protein-MAPK pathways in cardiac myocytes, smooth muscle, and COS cells (31–33). In avian lymphoma cells, we found multiple tyrosine kinases involved in G protein-MAPK pathways. In PC12 cells, Pyk2 is proposed to work together with Src to link G protein-coupled receptors to MAPK (34). The epidermal growth factor receptor, a receptor tyrosine kinase, has been implicated in transducing signals from G protein-coupled receptors to the MAPK cascade in Rat-1 cells (73). Another receptor tyrosine kinase, the platelet-derived growth factor receptor, has also been indicated to be involved in angiotensin II receptor-mediated mitogenic signaling in vascular smooth muscle cells (74). Therefore, different tyrosine kinases mediate the G protein signals in a general or cell type-specific way. However, the usage of tyrosine kinases in the G protein-MAPK pathway in some cells does not rule out a role for other tyrosine kinase-independent pathways in other cells. In fact, there is some evidence that in certain cells activation of the MAPK cascade by certain G protein-coupled receptors was not sensitive to tyrosine kinase inhibitors (21, 27, 36). Here we want to stress again the specificity of signaling pathways depends on the cellular environment, which is a very familiar theme to developmental biologists.

How do nonreceptor tyrosine kinases activate the MAPK cascade? At this point, we can only speculate that Syk, like receptor tyrosine kinases, can recruit adapter molecules such as SHC to transmit signals to the MAPK cascade (15–17). Several recent reports have suggested that tyrosine phosphorylation of SHC and the subsequently recruited Grb2-SOS complex are involved in activation of MAPK by G\(_{\alpha}\) and G\(_{\beta}\)-coupled receptors in a RAS-dependent pathway (28, 29, 75). Furthermore, Syk has been shown to associate with SHC (58). This is very similar to the mechanism proposed for FAK in linking the adapter protein Grb2 to RAS and MAPK activation in integrin signaling (60).
In comparison of the yeast and vertebrate G protein-MAPK pathways, although both use protein kinases to link G proteins to the MAPK pathway, yeast utilizes serine/threonine protein kinase STE20, while in DT40 cells the linker is a tyrosine kinase cascade (Fig. 6B). Recent evidence suggests that the small GTP-binding protein CDC42 is required in the yeast pheromone response pathway (76, 77). Another small GTP-binding protein RAS has also been shown to be required for the fission yeast Schizosaccharomyces pombe pheromone response pathway (11) and for some G protein-coupled receptor-MAPK pathways in vertebrates (19–21). Future experiments with knock-out DT40 cells will be necessary to determine any essential roles for CDC42, RAS, Gq- or Gi-coupled MAPK pathways.

Acknowledgments—We thank Dr. P. Low for the plasmid pCDB3/ T7–7 and L. Chen for purified NF-AT-His6 protein. We are grateful to our colleagues and the members of our laboratory for reading the manuscript.

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