JNK-Interacting Leucine Zipper Protein Is a Novel Scaffolding Protein in the $G\alpha_{13}$ Signaling Pathway†

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ABSTRACT: Scaffolding proteins play a critical role in conferring specificity and fidelity to signaling pathways. The JNK-interacting leucine zipper protein (JLP) has been identified as a scaffolding protein involved in linking components of the JNK signaling module. $G\alpha_{12}$ and $G\alpha_{13}$, the α-subunits of heterotrimeric G proteins G12 and G13, respectively, stimulate the JNK module in diverse cell types. Here, we report that $G\alpha_{13}$ physically interacts with JLP, and this interaction enhances $G\alpha_{13}$-mediated JNK activation. We also demonstrate endogenous interaction between JLP and $G\alpha_{13}$ in MCF-7 cells. JLP interaction is specific to the G12 family of α-subunits via its C-terminal domain (termed GID-JLP), spanning amino acids 1165−1307, and this interaction is more pronounced with the mutationally or functionally activated form of $G\alpha_{13}$ compared to that of wild-type $G\alpha_{13}$. The presence of a ternary complex consisting of $G\alpha_{13}$, JLP, and JNK suggests a role for JLP in tethering $G\alpha_{13}$ to the signaling components involved in JNK activation. Coexpression of GID-JLP disrupts ternary complex formation in addition to attenuating $G\alpha_{13}$-stimulated JNK activity. These findings identify JLP as a novel scaffolding protein in the $G\alpha_{13}$-mediated JNK signaling pathway.

Cell signaling involves dynamic networking between the signaling pathways that overlap, cross talk, and/or antagonize each other (*1*, *2*). In many instances, a similar or identical set of signaling molecules are exchanged between different pathways to elicit functionally distinct responses. Defining the mechanism by which an individual pathway is insulated from the "noise" or the "signal drifting" from the adjacent signaling pathways is, thus, of crucial interest. The scaffolding function of several newly identified proteins appears to provide such a mechanism by tethering a variety of signaling molecules in a cell- and context-specific manner. $G\alpha_{12}$ and $G\alpha_{13}$, the α-subunits of the G12 family of heterotrimeric G proteins,[1] differentially regulate a number of MAP kinases, which, in turn, elicit a multitude of cellular responses required for different physiological stimuli (*3*, *4*). The signaling complexity and precision of $G\alpha_{12}$ and $G\alpha_{13}$ indicate the possible involvement of a scaffolding protein that can modulate specific signaling responses. Although earlier studies with *Saccharomyces cerevisiae* have identified the critical role of the scaffolding protein Ste5p, in G protein *Gpa* signaling to the Ste20 kinase module (*5*), such scaffolding proteins that can tether mammalian heterotrimeric G proteins to the kinase signaling module have not yet been identified.

Recently, we have identified JLP (JNK-associated leucine zipper protein) as a new member of the family of JNK-interacting proteins (JIPs), which provides a scaffolding function for the JNK/p38MAPK signaling module (*6*). JLP is encoded by the *JIP4* gene from which three distinct splice variants, namely, JLP, JIP4, and SPAG9, are generated (*7*). Unlike other JIP family members (including JIP4), JLP is expressed ubiquitously, thereby suggesting a more generalized role in the regulation of the JNK in many different tissues (*6*−*10*). Since $G\alpha_{12}$ or $G\alpha_{13}$ potently activates the JNK module in a variety of cell types (*3*), we sought to determine whether JLP can act as a scaffold to tether $G\alpha_{12}$ or $G\alpha_{13}$ to the JNK module. Here, we present our results demonstrating that JLP is a novel scaffolding protein that links $G\alpha_{12}$ or $G\alpha_{13}$ to the JNK-signaling module.

EXPERIMENTAL PROCEDURES

Cells, Plasmids, and Transfections. COS-7 and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Life Technologies, Inc., Carlsbad, CA) containing 10% FBS (Invitrogen Life Technologies) and 1% penicillin/streptomycin at 37 °C in a 5% CO_2 incubator. Expression vectors encoding S-epitope-tagged JLP and its mutant derivatives (*6*) as well as different Gα subunits have been previously described (*11*). Transfection of COS-7 cells was carried out using FuGENE-6 reagent (Roche Applied Sciences, Indianapolis, IN) according to the manufacturer's protocol.

Coprecipitation and Immunoblot Analysis. Coprecipitation studies were carried out with COS-7 cells using S-protein−

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[1] Abbreviations: G protein, guanine nucleotide binding protein; JLP, JNK-interacting leucine zipper protein; GID, $G\alpha_{13}$-interacting domain; JNK, c-Jun NH_2-terminal kinase; MAP kinase, mitogen-activated protein kinase; JIP, JNK-interacting protein; JSAP, JNK/stress-activated protein kinase-associated protein; GTP, guanine triphosphate; GEF, guanosine nucleotide exchange factor; WT, wild type; LZ, leucine zipper; HA, hemagglutinin; AlF_4, aluminum fluoride.

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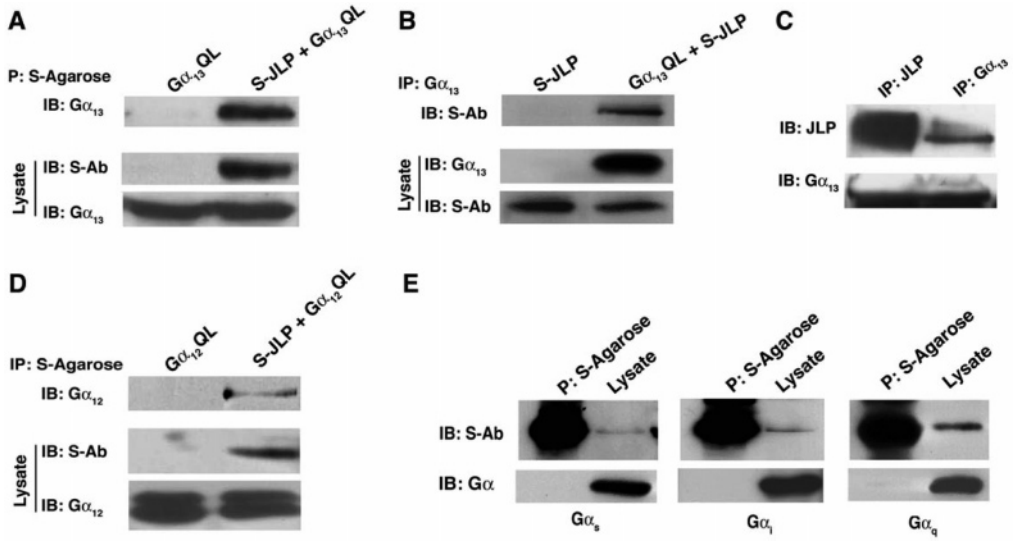

FIGURE 1: Specific interaction between JLP and the G12 family of G proteins. (A) Lysates from COS-7 cells expressing $G\alpha_{13}$QL along with S-epitope tagged JLP or its empty vector were subjected to the S-protein−agarose pull-down assay as described in Experimental Procedures. Coprecipitated $G\alpha_{13}$ was detected by immunoblot analysis using a $G\alpha_{13}$ antibody. Whole cell lysates were subjected to immunoblot analysis with antibodies to $G\alpha_{13}$ and S-tag, to monitor the expression levels of the respective proteins. (B) Lysates of COS-7 cells coexpressing $G\alpha_{13}$QL along with S-epitope-tagged JLP were immunoprecipitated using antibodies to $G\alpha_{13}$. The presence of JLP in the $G\alpha_{13}$ immune complex was detected using the S-tag antibody. Cell lysates were subjected to immunoblot analyses with antibodies to $G\alpha_{13}$ and S-tag to monitor the expression levels of the respective proteins. (C) Association between $G\alpha_{13}$ and JLP in situ was monitored by immunoprecipitation using either $G\alpha_{13}$ or JLP from the lysates prepared from MCF-7 cells using antibodies to $G\alpha_{13}$ or JLP, respectively. $G\alpha_{13}$ in the JLP immune complex and JLP in the $G\alpha_{13}$ immune complex were detected by immunoblot analysis using the respective antibodies. (D) COS-7 cells expressing S-tagged JLP or empty vector were cotransfected with $G\alpha_{12}$QL. Lysates were subjected to the S-agarose pull-down assay followed by immunoblot analysis with the $G\alpha_{12}$ antibody. (E) $G\alpha_s$QL, $G\alpha_i$QL, or $G\alpha_q$QL was cotransfected along with S-JLP into COS-7 cells. Following the S-agarose pull-down assay, precipitates were examined for the presence of $G\alpha_s$, $G\alpha_i$, or $G\alpha_q$ by immunoblot analyses.

agarose beads (Novagen, EMD Biosciences, Inc., Madison, WI) or antibodies specific to proteins of interest. Twenty-four hours following transfection, cells were lysed and the cell lysate protein (500 μg each) was incubated with 30 μL of S-protein−agarose beads for 4 h at 4 °C. After repeated washes with lysis buffer, the S-protein−agarose-bound proteins were separated by SDS−PAGE and electroblotted onto PVDF membranes. Co-immunoprecipitation analyses were carried out by incubating cell lysate protein (500 μg each) with 1 μg of the respective antibodies for 4 h at 4 °C followed by the addition of 20 μL of a 50% slurry of protein A−Sepharose (Amersham Biosciences Corp., Piscataway, NJ). Antibodies to the HA epitope (2362) and phosphorylated JNK (9251) were obtained from Cell Signaling Solutions (Beverly, MA), whereas antibodies to the S epitope (sc-802), JNK-1 (sc-474), $G\alpha_s$ (sc-823), $G\alpha_q$ (sc-393), and $G\alpha_{12}$ (sc-409) were obtained from Santa Cruz Biotechnology Inc., (Santa Cruz, CA). Antibodies to $G\alpha_i$ (1521) were a kind gift from D. Manning (University of Pennsylvania, Philadelphia, PA). For immunoprecipitation as well as immunoblot analysis for $G\alpha_{13}$WT and $G\alpha_{13}$QL, rabbit polyclonal antibodies (AS1-89-2) raised against the C-terminus of $G\alpha_{13}$ were used. After the immunoprecipitates had been washed twice with lysis buffer, the immunoprecipitated proteins were separated by SDS−PAGE and electroblotted onto PVDF membranes. Immunoblot analyses with specific antibodies were carried out following the previously published procedures (*4*).

RESULTS

Interaction between $G\alpha_{13}$ and JLP. Our previous studies along with others have shown that $G\alpha_{13}$ activates JNK (*12*, *13*), and since JLP is involved in assembling the JNK signaling module (*6*), the potential interaction between these two proteins was examined. To determine whether $G\alpha_{13}$ interacts with JLP, pull-down studies were carried out using COS-7 cells that were transiently transfected with S-tagged JLP (S-JLP) along with the constitutively activated mutant of $G\alpha_{13}$ ($G\alpha_{13}$QL). Lysates from these transfectants were subjected to the pull-down assay using S-protein−agarose beads. When the precipitates were analyzed for the presence of $G\alpha_{13}$ by immunoblot analysis with $G\alpha_{13}$-specific antibodies, $G\alpha_{13}$ was detected in S-JLP immunoprecipitates (Figure 1A). Similarly, JLP was detected in $G\alpha_{13}$ immunoprecipitates by protein immunoblot analysis (Figure 1B). Together, these results demonstrate a physical interaction between JLP and $G\alpha_{13}$.

To rule out the possibility that the observed interaction is due to ectopic expression of $G\alpha_{13}$ and JLP, we examined this interaction in a cell line known to express both proteins. Such an interaction between JLP and $G\alpha_{13}$ in situ can be demonstrated in MCF-7 breast cancer cells. When JLP was immunoprecipitated from the cell lysates, the presence of associated enodogenous $G\alpha_{13}$ was detected in the precipitate (Figure 1C). Likewise, immunoprecipitation of $G\alpha_{13}$ from MCF-7 cell lysates illustrates the presence of associated endogenous JLP in $G\alpha_{13}$ immunoprecipitates (Figure 1C). Together, these results indicate that the interaction between JLP and $G\alpha_{13}$ occurs even under conditions in which these proteins are not overexpressed.

Next, we investigated the specificity of the $G\alpha_{13}$−JLP interaction. Since $G\alpha_{12}$ is closely related to $G\alpha_{13}$, with 67% of their amino acids being identical, and is the only other member of the G12 family of G proteins (*14*), we examined whether JLP interacts with $G\alpha_{12}$. COS-7 cells were cotransfected with vectors encoding S-JLP and $G\alpha_{12}$QL, and the

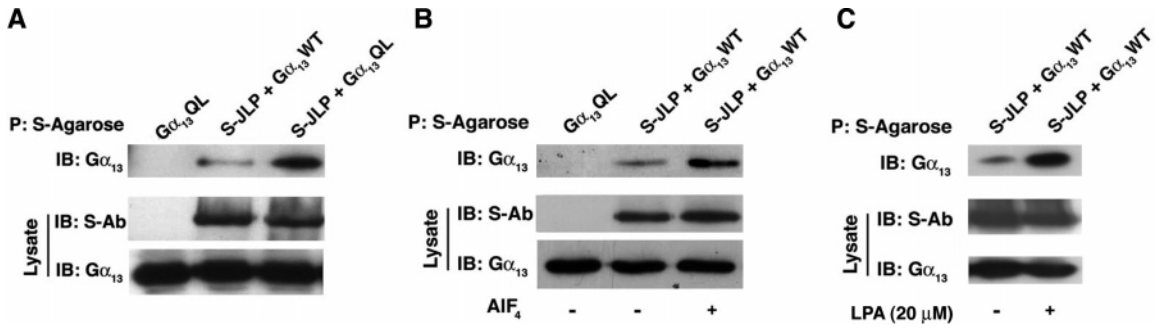

FIGURE 2: Interaction of JLP with WT vs constitutively active $G\alpha_{13}$. (A) COS-7 cells were cotransfected with S-JLP along with $G\alpha_{13}$WT or $G\alpha_{13}$QL. Lysates were collected, and the pull-down assay was carried out using S-agarose beads. Immunoblot analysis was performed with both precipitates and lysates using $G\alpha_{13}$ antibody along with S-antibody. (B) COS-7 cells were cotransfected with vectors encoding S-JLP and $G\alpha_{13}$WT. Transfectants were preincubated with 30 μM $AlCl_3$ and 10 mM NaF for 15 min ($+AlF_4$) prior to lysis along with nontreated control group ($-AlF_4$). The $G\alpha_{13}$ subunit that coprecipitated along with S-JLP by S-agarose was identified by immunoblot analysis using $G\alpha_{13}$ antibodies. Expression levels of JLP and $G\alpha_{13}$ in these transfectants were monitored by immunoblot analyses with respective antibodies. (C) COS-7 cells were cotransfected with vectors encoding S-JLP and $G\alpha_{13}$WT. Transfectants were serum-starved for 24 h followed by 20 μM LPA stimulation for 10 min. Expression levels of JLP and $G\alpha_{13}$ in these transfectants were monitored by immunoblot analyses with respective antibodies.

lysates were subjected to a pull-down assay with S-agarose beads as described above. The precipitated S-agarose beads were analyzed for the presence of S-JLP-associated $G\alpha_{12}$. Results from such analysis indicated that while there is an interaction, this JLP−$G\alpha_{12}$ interaction is not as potent as the JLP−$G\alpha_{13}$ interaction (Figure 1D). When similar analyses were carried out to determine whether JLP interacted with additional α-subunits belonging to the Gs, Gi, or Gq family of G proteins, the results indicated that JLP did not interact with any other Gα subunits (Figure 1E). Thus, our results illustrate that JLP interacts specifically with the α-subunits of the G12 family of G proteins (Figure 1A−E).

Activation of $G\alpha_{13}$ Potentiates Its Interaction with JLP. $G\alpha_{12}$QL and $G\alpha_{13}$QL mutants are deficient in their intrinsic GTPase activity and therefore are persistently in a GTP-bound, constitutively active, conformation (*3*). Thus far, our results have shown that the constitutively active mutants of $G\alpha_{12}$ and $G\alpha_{13}$ interact with JLP. To test whether such an active conformation of $G\alpha_{12}$ or $G\alpha_{13}$ is required for interaction with JLP, we analyzed the interaction of wild-type $G\alpha_{13}$ with JLP in comparison with that of its activated mutant. COS-7 cells were cotransfected with S-JLP and either the active (QL) or the wild-type (WT) form of $G\alpha_{13}$. The S-agarose pull-down assay followed by immunoblot analysis with $G\alpha_{13}$ antibody indicated that $G\alpha_{13}$QL, the activated form of $G\alpha_{13}$, binds with higher affinity to JLP than the WT form (Figure 2A). Furthermore, pretreatment of $G\alpha_{13}$WT transfectants with aluminum fluoride for 15 min, which is known to convert the unstimulated wild-type α-subunit to an active conformation (*11*, *15*, *16*), enhanced the interaction of JLP with $G\alpha_{13}$WT (Figure 2B). In addition, activation of $G\alpha_{13}$-WT by LPA (lysophosphatidic acid) similarly enhanced the interaction between $G\alpha_{13}$ and JLP (Figure 2C). Taken together, these findings indicate that the activated $G\alpha_{13}$ interacts more avidly with JLP (Figure 2).

$G\alpha_{13}$ Interaction Is Mediated by the C-Terminal Domain of JLP. Having established the participation of JLP in the $G\alpha_{13}$−JNK signaling module, we next focused on identifying the binding domain of JLP involved in interacting with $G\alpha_{13}$. To define the region within JLP responsible for interaction with $G\alpha_{13}$, a series of S-tagged JLP mutants truncated at the C-terminus were cotransfected with $G\alpha_{13}$QL (Figure 3A). Our initial findings indicated that the primary interaction between JLP and $G\alpha_{13}$ involves the C-terminal domain of JLP spanning amino acids 1000−1307 (Figure 3B). However, weaker interactions between other regions of JLP and $G\alpha_{13}$ can also be observed. It should be noted that JLP could form homodimers through its N-terminus (C. M. Lee and E. P. Reddy, unpublished data). It is, thus, likely that the transfected S-JLP mutants containing intact N-termini but truncated C-termini form homodimers with endogenous JLP. Therefore, the apparent weaker interactions seen with some of these mutants (Figure 3B) are possibly due to the interactions between the homodimerized endogenous JLP that associates with $G\alpha_{13}$ through their intact C-termini. Consequently, we decided to further define the interaction between the C-terminal domains of JLP with $G\alpha_{13}$, using domain mutant constructs of JLP encoding different regions of JLP, but lacking the JLP-dimerizing region (Figure 3D). Since these constructs would not contain the N-terminal JLP-interacting region including the LZI and LZII domains, the potential identification of spurious interactions would be minimal.

COS-7 cells were cotransfected with $G\alpha_{13}$QL together with these S-tagged domain mutants of different JLP regions. Lysates from these transfectants were subjected to the S-agarose pull-down assay. We observed that $G\alpha_{13}$ interacted only with the extreme C-terminal domain containing amino acids 1165−1307 (Figure 3E), thus confirming the results observed with the C-terminally truncated mutants (Figure 3B). Together, results from these studies suggest that the C-terminal domain of JLP spanning amino acids 1165−1307 defines the $G\alpha_{13}$-interacting domain (GID) of JLP.

JLP-Mediated Ternary Complex Involving JNK and $G\alpha_{13}$. Having established that a physical association exists between $G\alpha_{13}$ and JLP, we next attempted to determine if such an association has functional or regulatory consequences in JNK signaling. For this, COS-7 cells were cotransfected with hemagglutinin epitope-tagged JNK (HA-JNK), S-JLP, and $G\alpha_{13}$QL. The lysates from these transfectants were incubated with S-agarose beads, and immunoblot analysis was carried out with antibodies either to JNK or to $G\alpha_{13}$. Results from this study indicated that JLP associates with both JNK and $G\alpha_{13}$ (Figure 4A). The observed results could be indicative of the presence of a ternary complex involving JLP, $G\alpha_{13}$, and JNK or two different binary complexes, one involving JLP and $G\alpha_{13}$ and the other involving JLP and JNK.

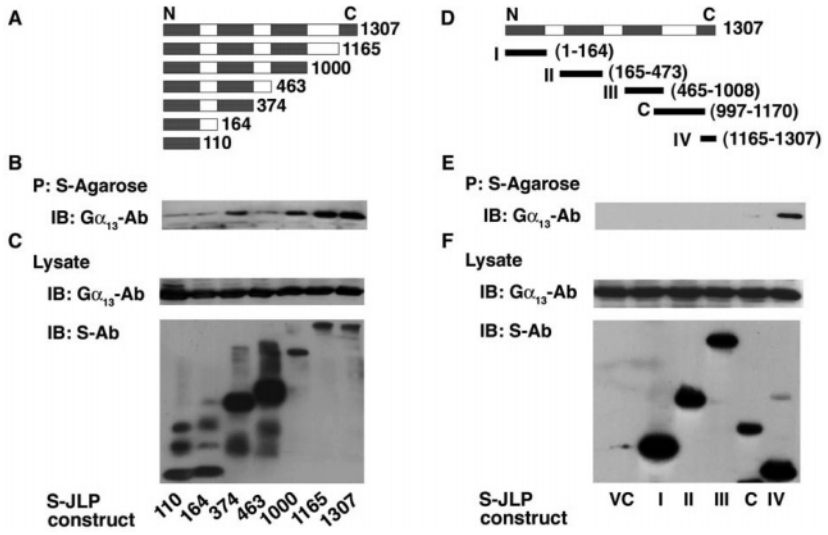

FIGURE 3: Deletion mapping of the $G\alpha_{13}$-interacting domain of JLP. (A) Schematic diagram of the C-terminally truncated S-JLP constructs used for analysis of the JLP−$G\alpha_{13}$ interaction. These mutants were 110 (amino acids 1−110), 164 (amino acids 1−164), 374 (amino acids 1−374), 467 (amino acids 1−467), 1000 (amino acids 1−1000), 1165 (amino acids 1−1165), and 1307 (full length, residues 1−1307). (B) $G\alpha_{13}$QL was coexpressed with full-length or C-terminally truncated mutants of S-JLP in COS-7 cells. The lysates were subjected to the pull-down assay with the S-protein−agarose beads. The coprecipitated $G\alpha_{13}$ was identified by immunoblot analysis using antibodies to $G\alpha_{13}$. (C) Immunoblot analyses with antibodies to $G\alpha_{13}$ or S-tag antibodies were carried out to monitor the expression of the transfected $G\alpha_{13}$ (top panel) and S-JLP constructs (bottom panel). (D) Schematic diagram representing JLP mutant constructs encoding different domains. These mutants were JLP-I-S (amino acids 1−164), JLP-II-S (amino acids 165−473), JLP-III-S (amino acids 465−1008), JLP-C-S (amino acids 997−1170), and JLP-IV-S (amino acids 1165−1307). (E) $G\alpha_{13}$QL was coexpressed with the different mutants of S-JLP in COS-7 cells. The lysates were subjected to the pull-down assay with the S-protein−agarose beads. The coprecipitated $G\alpha_{13}$ was identified by immunoblot analysis using antibodies to $G\alpha_{13}$. (F) Immunoblot analyses with antibodies to $G\alpha_{13}$ or S-tag were carried out to monitor the expression of the transfected $G\alpha_{13}$ (top panel) and S-JLP constructs (bottom panel).

To demonstrate that JLP indeed facilitates the formation of a ternary complex containing $G\alpha_{13}$, JLP, and JNK, the HA-tagged JNK in these lysates was immunoprecipitated with antibodies to the HA epitope. The immunoprecipitates were subjected to immunoblot analysis with antibodies to S-tag and $G\alpha_{13}$ to detect JLP and $G\alpha_{13}$, respectively. Results from these studies confirmed the previous finding that JLP associates with JNK (*9*). In the absence of JLP, JNK failed to interact with $G\alpha_{13}$ as anticipated (Figure 4B, lane 2). Since $G\alpha_{13}$ is not known to interact with JNK, and neither of these proteins contains any specific motifs for such an interaction, the absence of an interaction between $G\alpha_{13}$ and JNK is not surprising. However, when JLP was coexpressed in these cells, the interaction between $G\alpha_{13}$ and JNK could be observed readily (Figure 4B, lane 3). These results suggest the formation of a ternary complex involving JLP, JNK, and $G\alpha_{13}$ only in the presence of JLP. Previously published data have revealed that two different regions in the N-terminus of JLP are required for interaction with JNK (*9*). The data presented here show that the C-terminus of JLP (GID) is involved in interacting with $G\alpha_{13}$. Therefore, our findings indicate that JLP interacts with JNK and $G\alpha_{13}$ through distinct nonoverlapping domains.

As our results identify amino acids 1165−1307 as the $G\alpha_{13}$-interacting domain of JLP (GID-JLP), it can be speculated that the overexpression of this domain would disrupt JLP and $G\alpha_{13}$ interaction and essentially could serve as a dominant negative mutant for $G\alpha_{13}$−JLP interaction and function. To substantiate the scaffolding role of JLP further in this ternary complex, we investigated whether the coexpression of GID-JLP would inhibit such ternary complex formation by JLP. As the association between $G\alpha_{13}$ and JNK occurs only in the presence of JLP (Figure 4B), the presence of $G\alpha_{13}$ in JNK immunoprecipitates was used as an index for JLP-mediated ternary complex formation. COS-7 cells were transiently cotransfected with $G\alpha_{13}$ and HA-tagged JNK along with S-tagged full-length JLP, GID-JLP, or full-length JLP with GID-JLP. The HA-tagged JNK in these lysates was immunoprecipitated using HA antibody, and $G\alpha_{13}$ in the immunoprecipitates was detected by immunoblot analysis using antibodies to $G\alpha_{13}$. The presence of $G\alpha_{13}$ in HA immunoprecipitates of the transfectants expressing full-length JLP, HA-JNK, and $G\alpha_{13}$QL indicated the formation of a ternary complex (Figure 4C, top panel, lane 3). However, the coexpression of GID-JLP inhibited this complex formation (Figure 4C, top panel, lane 5), confirming the mandatory role of JLP in bringing this complex together.

Considering our previous observations that $G\alpha_{13}$QL stimulates JNK activity (*4*), we next sought to determine whether this complex also enhances the activation of JNK. To identify such a functional connection, JNK activity was examined in the lysates. The activation profiles of JNK were monitored by immunoblot analysis using antibodies to phosphorylated JNK. Our results show that the expression of $G\alpha_{13}$ is able to increase JNK activity (Figure 4C, bottom panel, lane 2). However, coexpression of JLP further enhances the activation of JNK by $G\alpha_{13}$ (Figure 4C, bottom panel, lane 2 vs lane 3). Since GID-JLP is shown to inhibit this ternary complex formation, it can be anticipated that its coexpression would inhibit $G\alpha_{13}$-mediated activation of JNK by sequestering $G\alpha_{13}$. Consistent with this notion, the coexpression of GID-JLP inhibited the activation of JNK by JLP and $G\alpha_{13}$ (Figure 4C, bottom panel, lanes 4 and 5). Thus, our results indicate that by inhibiting the formation of ternary complex, GID-JLP is able to inhibit JNK activation.

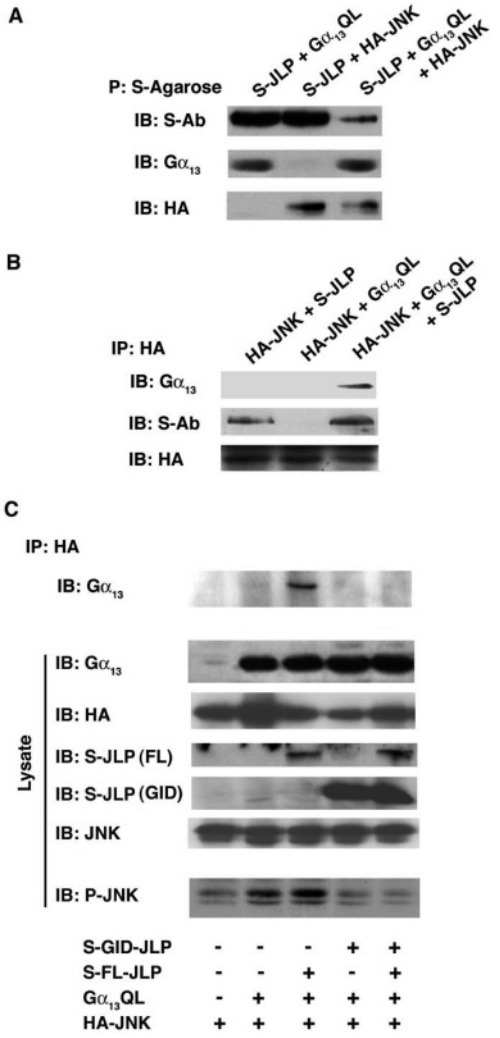

FIGURE 4: Analysis of JLP-mediated ternary complex formation. COS-7 cells were cotransfected with expression vectors encoding S-JLP, HA-JNK, and $G\alpha_{13}$QL. Cell lysates were examined through pull-down assays with S-agarose beads or immunoprecipitation using HA antibody. (A) Coprecipitated $G\alpha_{13}$ and HA-JNK in S-JLP precipitate were monitored with the corresponding specific antibodies against HA-tag, S-tag, and $G\alpha_{13}$. (B) Co-immunoprecipitated $G\alpha_{13}$ and JLP in HA-JNK immunoprecipitates were monitored using specific antibodies to HA-tag, S-tag, and $G\alpha_{13}$. (C) COS-7 cells were cotransfected with JNK, $G\alpha_{13}$QL, and full-length S-JLP or mutant GID-JLP (bearing amino acids 1165−1307). Lysates were analyzed by immunoprecipitation with HA antibody and analyzed for the presence of $G\alpha_{13}$ using $G\alpha_{13}$ antibody (top panel). Total cell lysates were analyzed for JNK phosphorylation using phospho-JNK antibody (bottom panel). Cell lysates were also analyzed for the expression of HA-JNK, $G\alpha_{13}$QL, full-length S-JLP, and mutant GID-JLP with their respective antibodies (middle panels).

LPA−$G\alpha_{13}$ Stimulation of JNK Is Inhibited by GID-JLP. As shown In Figure 2C, LPA stimulates the interaction of $G\alpha_{13}$ and JLP. To establish the physiological role for this interaction, we investigated whether GID-JLP was able to inhibit LPA-mediated activation of JNK via $G\alpha_{13}$. To demonstrate, COS-7 cells were transiently cotransfected with $G\alpha_{13}$WT and HA-tagged JNK along with S-tagged full-length JLP, or GID-JLP. After 24 h, the transfectants were serum-starved for an additional 24 h, after which they were stimulated with 20 μM LPA for 10 min with appropriate unstimulated controls. The HA-tagged JNK in these lysates was immunoprecipitated, using HA antibody, and activation of JNK was detected by immunoblot analysis using antibodies to phospho-specific JNK antibodies. The results indicated that LPA stimulated the activation of JNK as evidenced by the hyperphosphorylated JNK that can be observed in $G\alpha_{13}$-WT and JNK transfectants (Figure 5). While the coexpression of JLP strengthened this response (Figure 5, lane 3 vs lane 7), GID-JLP drastically inhibited the ability of LPA to stimulate JNK activity (Figure 5, lane 8), suggesting the pertinent role of both JLP and LPA−LPAR $G\alpha_{13}$-mediated activation of JNK. From these observations, it can be concluded that JLP plays a key role in functional tethering of GPCR signaling to the JNK-signaling module.

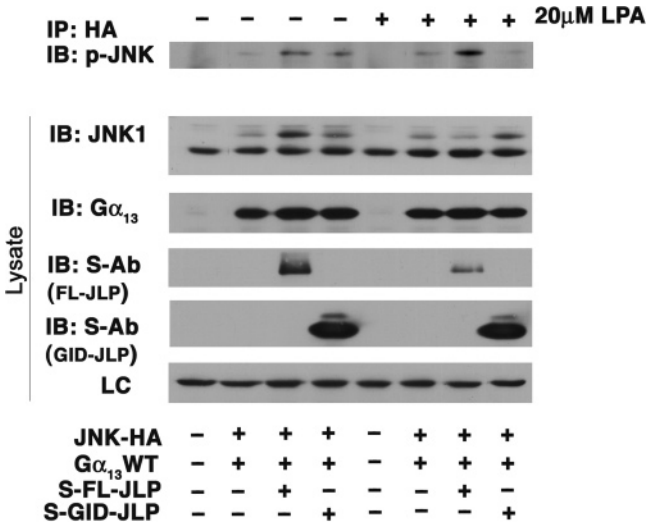

FIGURE 5: GPCR-mediated activation of JNK via the $G\alpha_{13}$−JLP complex. COS-7 cells were cotransfected with $G\alpha_{13}$WT, HA-JNK, and S-tagged full-length (FL) JLP or the C-terminal domain of JLP (GID). Twenty-four hours post-transfection, cells were serum-starved for an additional 24 h and stimulated with 20 μM LPA for 10 min. Lysates were immunoprecipitated with HA antibody. Immunoprecipitates were analyzed for JNK phosphorylation using phospho-JNK antibody. Expression levels of JNK, $G\alpha_{13}$, full-length JLP, and GID-JLP were monitored by immunoblot analysis with the respective antibodies.

DISCUSSION

Mitogen-activated protein kinase pathways, consisting of the three-tier kinase signaling modules, have been shown to interact with JNK scaffolding proteins. Although heterotrimeric G proteins such as Gi, Gq, and G12/13 have been shown to activate these modules, the identity of a specific scaffolding protein that can tether them to MAPK signaling modules is hitherto unknown. Our observation presented here demonstrating a novel interaction between the JNK scaffolding protein JLP and $G\alpha_{13}$ identifies, for the first time, the role for a JNK scaffolding protein in a G protein signaling pathway. Among the different subfamilies of G proteins, G12/13 has been observed to be the most potent in activating the JNK signaling pathway. Our observation presented here showing that JLP specifically interacts with $G\alpha_{12}$ and $G\alpha_{13}$ and not with $G\alpha_s$, $G\alpha_i$, or $G\alpha_q$ points to the critical role of JLP in $G\alpha_{12}$- and $G\alpha_{13}$-mediated activation of the JNK signaling pathway. Since scaffolding proteins are known to accelerate the spatiotemporal kinetics of signaling events, our observation points to the unique ability of JLP to tether $G\alpha_{13}$ to the JNK signaling module, in a context-specific manner.

Our results also identify the C-terminal region of JLP, spanning amino acids 1165−1307, as the critical domain of interaction with $G\alpha_{13}$, termed as GID. Furthermore, we have shown that JLP facilitates the formation of a ternary complex containing $G\alpha_{13}$, JLP, and JNK. The finding that coexpression of JLP and $G\alpha_{13}$ enhances $G\alpha_{13}$-mediated JNK activation, whereas the overexpression of GID-JLP inhibits JNK activation, confirms the scaffolding role of JLP in $G\alpha_{13}$ signaling to the JNK module. Furthermore, the finding that LPA-stimulated activation of JNK is inhibited by the coexpression of GID stresses two interesting points: (1) GID can indeed disrupt signaling from LPA receptors to the JNK signaling module presumably by sequestering $G\alpha_{13}$, and (2) both $G\alpha_{13}$ and JLP are required for the full complement of JNK activation by LPA. Through these observations, we have identified, for the first time, a scaffolding protein that can assemble the α-subunit of a heterotrimeric G protein to a kinase-signaling module in a mammalian system.

Scaffolding proteins such as 14-3-3, β-arrestin, and JIPs, although not known to associate with G protein subunits, have been shown to link distinct proteins to multiple pathways (*1*). In many instances, it has been shown that these scaffolding proteins alter the spatiotemporal kinetics of signaling events. It has been shown $G\alpha_{12}$ and $G\alpha_{13}$ activate the JNK module by stimulating small GTPases Ras, Rac, and/or Rho via their respective GEFs (*12*, *17*, *18*). Considering the large number of candidate GEFs that could be involved (*19*−*21*), the study presented here does not address whether JLP tethers any of these GEFs to the JNK module. However, our initial studies point out that JLP interacts with the Rac/CDC42-specific exchange factor, β-Pix (K. Kashef and D. N. Dhanasekaran, data not shown). Taken together, the previous findings that β-PIX stimulates the GDP−GTP exchange activity of Rac and Rac stimulates the JNK module suggest the interesting possibility that JLP tethers $G\alpha_{13}$ to the downstream functional signaling unit involved in the activation of JNK.

The relationship of JLP with other $G\alpha_{13}$-interacting proteins is not clear at present. However, our observation that JLP does not associate with Hax-1 (D. Onesime and D. N. Dhanasekaran, unpublished observation), a $G\alpha_{13}$-interacting partner involved in cell motility (*11*), suggests that JLP tethers $G\alpha_{13}$ specifically to the JNK module. With these observations, one can speculate that JLP tethers $G\alpha_{13}$ to multiple signaling pathways in a context- or cell-type-specific manner. Aside from their scaffolding role in the JNK signaling pathway, the JIP/JLP class of proteins is known to interact with kinesin motor proteins (*7*). Kinesins are a family of motor proteins that are involved in cargo movement along microtubules (*25*, *26*). We have recently shown the requirement of kinesin for proper localization of JLP in the cellular domain (*27*). The observation that JLP is able to interact with both signaling and motor proteins has many implications. It can be envisaged that, in response to a signal, JLP binds to G protein in the plasma membrane and migrates—presumably, along with the critical downstream signaling components—toward the nucleus to facilitate the transactivation of the expression of specific genes, as depicted in Figure 6. Our previous observation that JLP translocates to the perinuclear region from the cytosol in response to stress-inducing reagents, such as UV and arsenite (*6*), attests to such a possibility. Whether JLP shows such signaling-dependent translocation to a specific cellular compartment in response to GPCR stimulation is presently being investigated in the laboratory.

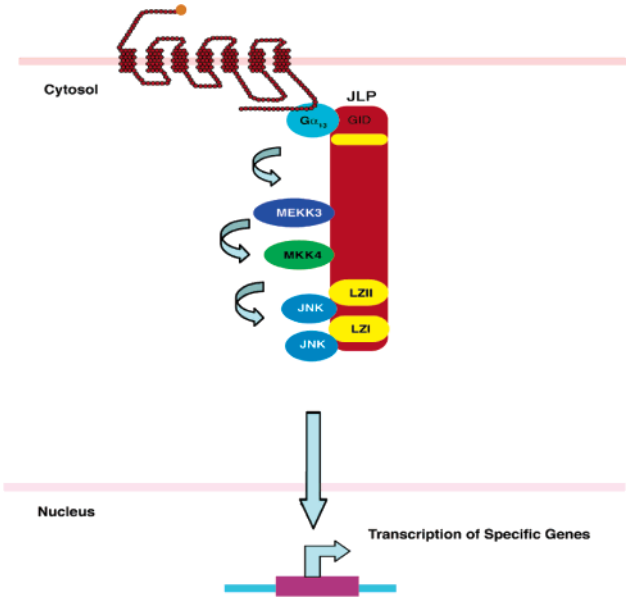

FIGURE 6: Proposed model for the $G\alpha_{13}$−JLP−JNK signaling pathway. In response to a signal, JLP binds to active $G\alpha_{13}$ in the plasma membrane. This scaffolding protein is also able to tether other specific downstream kinases and therefore facilitate signal transduction. Along with the critical downstream signaling components, JLP is able to move toward the nucleus to facilitate the transactivation of the expression of specific genes.

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