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Endodermal Differentiation of Murine Embryonic Carcinoma Cells by Retinoic Acid Requires JLP, a JNK-Scaffolding Protein

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Abstract Retinoic acid (RA) is a morphogen that induces endodermal differentiation of murine P19 embryonic carcinoma cells. RA-induced differentiation of P19 cells has been used as a model system to define the differentiation programs of pluripotent stem cells. Using this system it has been shown that $G\alpha_{13}$—the α-subunit of the heterotrimeric G protein G_{13}—and its activation of JNK-module are critically required for the endodermal differentiation of P19 cells. However, the mechanism through which $G\alpha_{13}$ is linked to JNK-module is unknown. Here, we report that RA stimulates the expression of JNK-interacting leucine zipper protein (JLP), a newly identified JNK-scaffolding protein and its critical role in RA-mediated endodermal differentiation. Our results indicate that there is a physical association between JLP and $G\alpha_{13}$ in RA-stimulated P19 cells. More interestingly, silencing JLP abrogates RA-mediated endodermal differentiation of P19 cells analogous to the effects seen with the silencing of $G\alpha_{13}$ or JNK. Therefore, our studies presented here identify for the first time, a novel role for a newly identified scaffolding protein in RA-mediated endodermal differentiation, providing a new signaling conduit to transmit signals from RA to JNK module. J. Cell. Biochem. 98: 715–722, 2006.

Key words: stem cell; G protein; JLP; JNK; endodermal differentiation; scaffolding protein; $G\alpha_{13}$

Stem cells are pluripotent in their ability to differentiate into multiple cell types depending upon extrinsic and intrinsic stimuli [Prelle et al., 2002; Wobus and Boheler, 2005]. Elucidating the signaling mechanisms underlying the commitment of these cells to a particular differentiation program has far-reaching implications towards the cure for many diseases [Prelle et al., 2002; Wobus and Boheler, 2005]. Murine P19 embryonic carcinoma cells have been widely used as a good model system to study stem cell differentiation. P19 cells undergo endodermal, mesodermal, or neuronal differentiation in response to different concentration of retinoic acid [Jones-Villeneuve et al., 1982]. Therefore, these cells have been widely used to decipher the signaling mechanisms underlying the specific differentiation programs. It has been shown that the differentiation of P19 cells towards primitive endodermal lineage, mediated by retinoic acid, involves $G\alpha_{13}$, the α-subunit of the heterotrimeric G protein G13 [Jho and Malbon, 1997; Jho et al., 1997; Kanungo et al., 2000].

Studies on the signaling pathways involved in RA-mediated differentiation have indicated that $G\alpha_{13}$ stimulates endodermal differentiation through the activation of JNK-signaling module [Jho and Malbon, 1997; Jho et al., 1997; Kanungo et al., 2000]. Consistent with this finding, the components of JNK-signaling module including MEKK-1, MEKK-4, and MKK-4 have been shown to be involved in this differentiation program [Jho and Malbon, 1997; Jho et al., 1997; Kanungo et al., 2000]. Recently, we have identified that JNK interacting Leucine Zipper protein (JLP), a scaffolding protein involved in assembling JNK-signaling module, interacts with $G\alpha_{13}$ [Kashef et al., 2005]. JLP belongs to the JNK-interacting protein (JIP) family of scaffolding proteins known for their

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ability to modulate the JNK kinase pathway by tethering the JNK-signaling module consisting of distinct MEKKs and MKKs [Lee et al., 2000]. Our studies have shown that JLP forms a ternary complex with $G\alpha_{13}$ and JNK to elicit the activation of JNK [Kashef et al., 2005]. It has also been shown that the expressions of $G\alpha_{13}$, as well as the constituent kinases of JNK-module, are upregulated during RA-mediated endodermal differentiation [Jho and Malbon, 1997; Jho et al., 1997; Kanungo et al., 2000]. Therefore, we sought out to investigate whether JLP plays a role in RA-mediated endodermal differentiation of P19 embryonal carcinoma cells. Here, we present our results demonstrating for the first time that (1) RA-treatment stimulates the expression of JLP, (2) JLP tethers $G\alpha_{13}$ in response to RA-treatment, and (3) JLP is essential for RA-mediated JNK activation and subsequent endodermal differentiation of P19 cells.

MATERIALS AND METHODS

Murine P19 Cell Cultures, Transfections, and Differentiation

P19 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Life technologies, Inc., Carlsbad, CA, USA) containing 10% FBS (Invitrogen Life technologies), and 1% penicillin/streptomycin at 37°C in a 5% CO_2 incubator. Endodermal differentiation of P19 cells was stimulated by treating the cells with 10^{-6} M retinoic acid (*all-trans*, Sigma) for 72 h.

Silencing JLP With Short Interfering RNAs (siRNA)

The coding region of murine JLP spanning 513–535 bases (5′-GGAACACTTAGAAAGAACCAAC-3′) was targeted for the synthesis of siRNA. The siRNA targeting this region was synthesized and purified (Qiagen Inc., Valencia, CA). An equimolar pool containing nonspecific control siRNA duplexes (# D-0011206-13-05, Dharmacon, Inc., Lafayette, CO) was used as control. Cells were transfected with 20 μM siRNA using TransMessenger reagent (Qiagen Inc., Valencia, CA) according to previously published procedures [Radhika et al., 2004].

Co-Precipitation and Immunoblot Analysis

Co-immunoprecipitation studies were carried out with P19 cells using specific antibodies to $G\alpha_{13}$, JLP, or JNK. P19 cells were lysed using previously published procedures [Lee et al., 2000; Radhika et al., 2004; Kashef et al., 2005] and cell lysate proteins (500 μg each) were incubated with 1 μg of respective antibodies for 4 h at 4°C followed by the addition of 20 μl of 50% slurry of protein A Sepharose (Amersham Biosciences Corp, Piscataway, NJ). Antibodies to phosphorylated-JNK (9251) were obtained from Cell Signaling Solutions (Beverly, MA) whereas antibodies to JNK-1 were obtained from Santa Cruz Biotechnology Inc., (Santa Cruz, CA). For immunoprecipitation as well as immunoblot analysis for $G\alpha_{13}$, rabbit polyclonal antibodies (AS1-89-2) raised against the C-terminus of $G\alpha_{13}$ were used [Radhika et al., 2004]. Likewise, immunoblot and immunoprecipitation studies for JLP was carried out using polyclonal antibodies raised against the C-terminus of JLP [Lee et al., 2000]. TROMA-1 (trophoblastoma antigen-1) antibody developed by Philippe Brulet and Rolf Kemler was obtained from the Developmental studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242. After washing the immunoprecipitates three times with lysis buffer, the immunoprecipitated proteins were separated by SDS–PAGE and electroblotted on to PVDF membranes. Immunoblot analyses with specific antibodies were carried out following the previously published procedures [Radhika et al., 2004; Kashef et al., 2005].

RT-PCR

An aliquot of the total RNA (2 μg) was converted into cDNA using ThermoScript RT-PCR System (Invitrogen Life Technologies, CA). The reverse transcribed cDNA was subjected to PCR with Taq PCR Core Kit (Qiagen Inc.).

The following primers were used for the RT-PCR [Choi et al., 2005]: α-Fetoprotein (AFP) specific forward (5′-AGTGCGTGACGGAGAAGAAT-3′) and reverse (5′-TGTCTGGAAGCACTCCTCCT-3′) primers; GATA-4 specific forward (5′-CTCCTACTCCAGCCCCTACC-3′) and reverse (5′-GTGGCATTGCTGGAGTTACC-3′) primers; The PCR reaction conditions were 2-min denaturation at 94°C followed by 30 cycles of 30 s. denaturation at 94°C, 1 min annealing at 58°C, and 1 min elongation at 72°C. PCR products were removed at 30 cycles.

The mouse GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase) specific forward (5′-GTGAAGGTCGGTTGTGAACGG-3′) and reverse (5′-GATGCAGGGATGATGTTCTG-3′) primers were used as control [Kumar et al., 2004]. The amplification products were analyzed by agarose gel electrophoresis.

RESULTS

Retinoic Acid Induces Endodermal Differentiation of P19 Cells

Previous studies have shown that *all-trans* RA induces the differentiation of murine P19 embryonic carcinoma stem cells to different lineages in a concentration dependent manner [McBurney, 1993]. While higher concentration (10^{-5} M) of RA has been shown to induce the neuronal differentiation of P19 cells, lower concentration (10^{-6} M) of RA has been shown to induce primitive endodermal differentiation [Jones-Villeneuve et al., 1982]. Endodermal differentiation of P19 cells can be monitored by the differentiated cell morphology or the expression of endodermal differentiation specific marker TROMA-1 [Kemler et al., 1981; Jho and Malbon, 1997; Jho et al., 1997]. Therefore, prior to embarking on studies to define the mechanism(s) underlying RA-mediated endodermal differentiation, we sought to validate RA-mediated endodermal differentiation in P19 cells. P19 cells were treated with 10^{-6} M RA for 72 h and analyzed under microscope to monitor RA-mediated endodermal differentiation. While control P19 cells have a cuboidal morphology and are densely packed, the RA treated cells showed differentiated phenotype specific to endodermal lineage. As shown in Figure 1A, the RA differentiated cells exhibit a much larger and spread-out morphology, characteristic of endodermal cells. Since it has been previously shown that the expression TROMA-1 is a specific marker for endodermal differentiation, lysates were prepared from RA-treated cells along with vehicle control, and subjected to immunoblot analysis to monitor its expression. In accordance with the expression profile of TROMA-1, control cells failed to express TROMA-1. In contrast, the RA-treated P19 cells showed robust expression of TROMA-1 validating the micrograph analysis (Fig. 1B).

Recently, the expression of GATA-4, a transcription factor, and α-fetoprotein (AFP), an embryonic plasma protein, has been identified to be specific for endodermal differentiation [Choi et al., 2005]. Therefore, to further validate that the P19 cells have acquired the endodermal phenotype, the expression levels of mRNA encoding these two endodermal markers were determined by RT-PCR in P19 cells differentiated with RA treatment. RNA from RA-treated and vehicle control P19 cells were used for reverse transcription [Kumar et al., 2004]. PCR was then carried out using primers specific to AFP and GATA-4; PCR with primers to GAPDH was used as control [Kumar et al., 2004; Choi et al., 2005]. As shown in Figure 1C, only the P19 cells treated with RA show the expression of both GATA-4 and AFP mRNA further validating the endodermal phenotype of these cells (Fig. 1C). Together, results from these analyses indicate that the treatment of P19 cells with 10^{-6} M RA induces the endodermal phenotype as indicated by the differential morphology and the expression of TROMA-1, GATA-4 and AFP marker (Fig. 1). As all of these three markers establish the endodermal differentiation of P19 cells upon RA treatment, taking into consideration that the expression of TROMA-1 has been widely used as a classic endodermal differentiation marker [Jho and Malbon, 1997; Wakayama et al., 2001], further studies to determine endodermal differentiation were carried out using the expression profile of TROMA-1.

Retinoic Acid Stimulates the Expression of JLP and $G\alpha_{13}$ in P19 Cells

Previously it has been shown that the endodermal differentiation of P19 cells critically require the expression of both $G\alpha_{13}$ and JNK [Jho and Malbon, 1997; Jho et al., 1997; Kanungo et al., 2000]. It has been shown that the treatment of P19 cells with 10^{-6} M RA stimulates the expression of $G\alpha_{13}$ and the resultant activation of JNK drives these cells towards endodermal differentiation [Jho and Malbon, 1997; Jho et al., 1997; Kanungo et al., 2000]. Our recent studies have shown that JLP provides the scaffolding function for $G\alpha_{13}$ to simulate JNK [Kashef et al., 2005]. Therefore, it can be reasoned that the RA-mediated endodermal differentiation requires a concomitant increase in the levels of JLP as well. To investigate, we examined whether RA also stimulates the expression of JLP during this differentiation process. P19 cells were treated with 10^{-6} M RA for 72 h and the

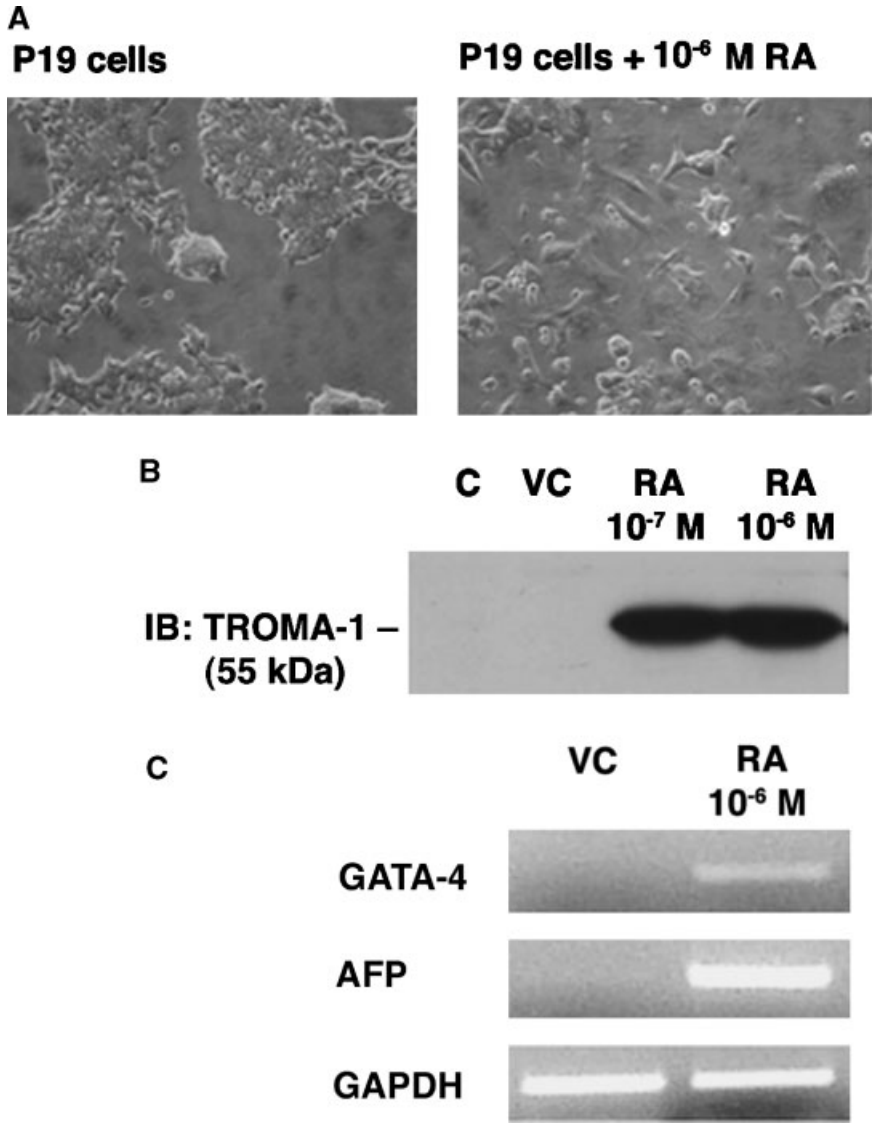

Fig. 1. Endodermal differentiation of P19 embryonal carcinoma cells with RA treatment. **A**: P19 cells were induced to endodermal differentiation by treatment with 10^{-6} M RA for 72 h. **B**: Undifferentiated (no RA) and fully differentiated (RA) P19 cells were harvested after 72 h. Whole cell lysates were subject to immunoblot analysis monoclonal antibody to the cytokeratin endo A (TROMA 1) marker protein for primitive endoderm. **C**: RA-treated endodermal differentiated cells and untreated P19 cells were collected and RNA extraction was carried out using Trizol Method. Two milligrams of RNA from the two samples was taken for reverse transcription. PCR was carried out with primers specific to AFP and GATA-4 and GAPDH using conditions described in the Material and Methods section. Lane IC: non-treated; Lane VC: vehicle (ethanol) treated.

expression levels of JLP were monitored along with $G\alpha_{13}$ and JNK in the cell lysates by immunoblot analysis using the respective antibodies. Results indicate that RA stimulates the expression of $G\alpha_{13}$ along with the activation of JNK as previously reported (Fig. 2) [Jho and Malbon, 1997; Jho et al., 1997]. The observation that RA-treatment enhances the expression of $G\alpha_{13}$ is consistent with the previous findings that RA stimulates a modest increase in $G\alpha_{13}$ levels [Jho and Malbon, 1997]. More interestingly, our results indicate, for the first time, that RA potently stimulates the expression of JLP (Fig. 2). It is highly significant that the expression of JLP, the scaffolding protein involved in the assembly of JNK-module, is increased during the endodermal differentiation of P19 cells. Our finding that JLP is highly expressed in the RA treated cells would suggest that the RA-

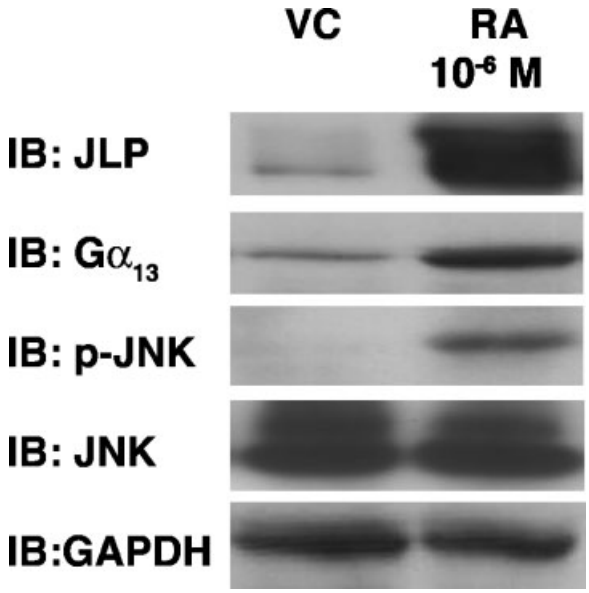

Fig. 2. RA stimulates JLP, $G\alpha_{13}$, and JNK activity. Endodermal differentiated P19 cells were collected along with undifferentiated cells. After 72 h, samples were lysed and immunoblot analysis was carried out to assess the levels of JLP, $G\alpha_{13}$, p-JNK, and JNK-1 using specific antibodies.

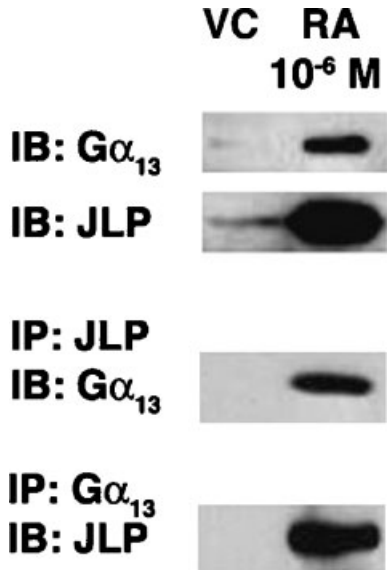

Fig. 3. Association between JLP and $G\alpha_{13}$ in P19 cells. JLP-$G\alpha_{13}$ interaction was monitored by immunoprecipitation using either $G\alpha_{13}$ or JLP from lysates of P19 cells (after 72 h of vehicle or RA treatment) using antibodies to $G\alpha_{13}$ or JLP, respectively. The presence of $G\alpha_{13}$ in JLP-immunocomplex and JLP in $G\alpha_{13}$-immunocomplex were detected by immunoblot analysis using the respective antibodies. Whole cell lysates were also subjected to immunoblot analyses with $G\alpha_{13}$ and JLP antibody to monitor expression levels.

mediated endodermal differentiation program requires JLP in this endodermal differentiation process.

Retinoic Acid Stimulates the Interaction Between JLP and $G\alpha_{13}$ in P19 Cells

Since (1) RA-mediated endodermal differentiation requires $G\alpha_{13}$ and JNK [Jho and Malbon, 1997; Jho et al., 1997], (2) RA stimulates the expression of $G\alpha_{13}$ and JLP along with the activation of JNK (Fig. 2), (3) JLP tethers $G\alpha_{13}$-JNK signaling module [Kashef et al., 2005], and (4) $G\alpha_{13}$ activates JNK [Vara Prasad et al., 1995; Dhanasekaran and Dermott, 1996; Voyno-Yasenetskaya et al., 1996; Arai et al., 2003], we investigated the potential interaction between JLP and $G\alpha_{13}$. To test whether JLP physically interacts with $G\alpha_{13}$, co-immunoprecipitation studies were carried out in P19 cells treated with RA. Lysates from these RA treated cells along with vehicle control group were subjected to pull-down assay using antibodies to JLP or $G\alpha_{13}$. When JLP was immunoprecipitated from the cell lysates, the presence of associated enodogenous $G\alpha_{13}$ was detected in the precipitate from RA-treated cells (Fig. 3). Likewise, when $G\alpha_{13}$ was immunoprecipitated from these lysates, the presence of associated endogenous JLP could be detected only in $G\alpha_{13}$-immunoprecipitates from RA-treated cells (Fig. 3). Together, these results indicate that in addition to stimulating the expression of $G\alpha_{13}$ and JLP, there is a physical interaction between JLP and $G\alpha_{13}$ in RA treated P19 cells.

Silencing JLP Abrogates Retinoic Acid-mediated Differentiation of P19 Cells

Thus far, our studies have shown that RA-mediated endodermal differentiation of P19 cells involves the upregulation of JLP, $G\alpha_{13}$, and JNK. Taken together with the previous findings, this would suggest that JLP mediates the signal transmission from RA to JNK-module by providing a scaffolding function. Previously it has been shown that the silencing of $G\alpha_{13}$ or inhibition of JNK abrogates RA mediated endodermal differentiation of P19 cells [Jho and Malbon, 1997; Jho et al., 1997]. Since JLP provides the scaffolding for JNK activation, we monitored the JNK activation profile in response to silencing of the JLP. The results from these experiments clearly indicate that the silencing of endogenous JLP using siJLP blunted JNK activation (Fig. 4A). After establishing the effect of siJLP in silencing JLP and the consequent reduction in the activity of JNK

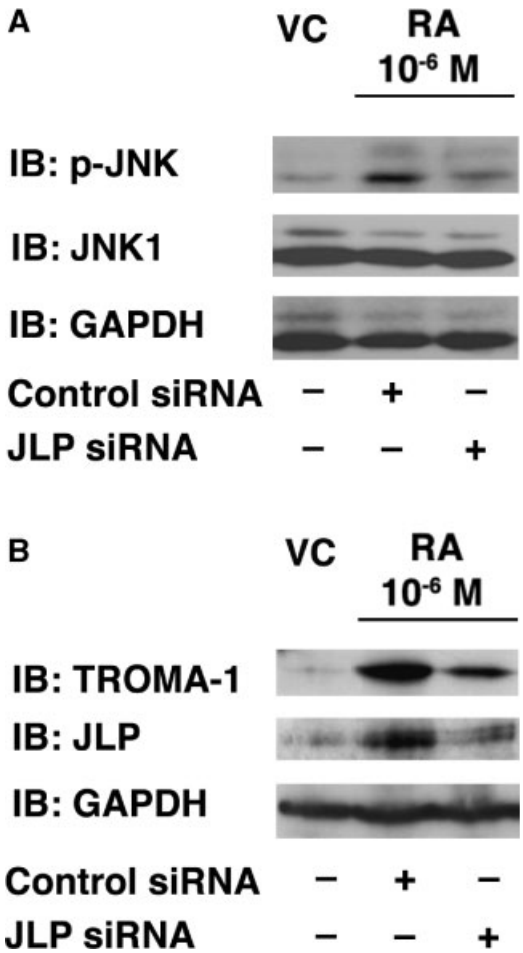

Fig. 4. Silencing JLP-expression in P19 cells inhibits JNK activation and endodermal differentiation. **A**: P19 cells were transfected with 20 μM scrambled non-specific control siRNA or siRNA targeted at murine JLP TransMessenger reagent (Qiagen Inc.). At 48 h, the cells were treated with 10^{-6} M RA for 72 h and lysed. The lysates were subjected to immunoblot analysis with antibodies specific to JLP, phospho-JNK and JNK1 to monitor their levels. To monitor loading control, the blots were stripped and reprobed with antibody raised against GAPDH. **B**: siJLP and non-specific control siRNA transfected P19 embryonal carcinoma cells were treated with RA for 72 h and lysed. Expression levels of endodermal marker TROMA-1 as well as JLP, $G\alpha_{13}$, and GAPDH levels were analyzed.

we analyzed the effect of siJLP on endodermal differentiation. To examine such a role for JLP, the expression of JLP in these cells were silenced using siJLP or control siRNA 48 h prior to 10^{-6} M RA treatment for 72 h. Samples were collected and the expression levels of TROMA-1 were monitored. Silencing JLP significantly inhibited the endodermal differentiation of P19 cells as shown in Figure 4B. Thus our results, for the first time, demonstrate a functional role and requirement for JLP in JNK activation and consequent RA-mediated endodermal differentiation of embryonic carcinoma cells.

DISCUSSION

Stem cells are a unique population of cells that have the ability to differentiate into a variety of tissue-specific cells. Defining the molecules that commit stem cells to a specific differentiation program is of critical importance in developing stem cell based therapies. The fate of the stem cell is highly dependent on both internal and external signals. One of the major challenges in stem cell biology is to identify the intracellular signaling mechanism that drive stem cells to specific specialized cell lineages. It has been shown that three-tier MAPK signaling modules provide one of the critical signaling mechanisms involved in these processes [Dhanasekaran and Premkumar Reddy, 1998; Garrington and Johnson, 1999]. It is interesting to note that these kinase modules are swapped between different pathways involved in differentiation as well as proliferation and apoptosis. Despite the presence of such overlapping array of signaling networks, a signal from a particular network is deciphered with great efficiency and precision without any cross-talk to other signaling networks. Recently, scaffolding proteins have been identified to tether different set of proteins in specific signaling network in a cell type and context specific manner. These scaffolding proteins include proteins such as 14-3-3, β-arrestins, and JNK interacting proteins (JIPs) [Garrington and Johnson, 1999; Morrison and Davis, 2003].

JIP family of scaffolding proteins consist of four family members including JIP1, 2, 3, and 4. They have been shown to be involved in mediating the JNK cascade through tethering different components of the JNK signaling module. Since the JNK signaling pathway is involved in several cellular functions including cellular proliferation, and differentiation the role of these scaffolding proteins has been of great interest recently. Of the different JIPs that have been identified, JLP, a splice variant of JIP4 [Morrison and Davis, 2003; Kelkar et al., 2005], has been identified to be involved in tethering $G\alpha_{13}$ to JNK-module [Kashef et al., 2005]. It should be noted here that JLP is the first scaffolding protein that has been shown to tether a kinase module to the heteroterimeric G

protein. We have also shown that JLP simultaneously tethers other proteins including JNK to provide a scaffold that brings these proteins into close proximity. In addition, we have also shown that JLP greatly potentiates $G\alpha_{13}$-mediated JNK activation [Kashef et al., 2005].

Previous studies have shown an obligatory role for $G\alpha_{13}$-stimulated JNK in RA-mediated endodermal differentiation program. It has been shown that the silencing of $G\alpha_{13}$ or mutational disruption of JNK abrogates RA-mediated endodermal differentiation [Jho and Malbon, 1997]. Taken together with our recent observation that $G\alpha_{13}$ stimulation of JNK involves the scaffolding function of JLP [Kashef et al., 2005], our present finding that RA stimulates $G\alpha_{13}$-JLP interaction along with an enhanced activation of JNK (Fig. 2) is highly significant. It should be noted here that JLP interacts more avidly with the GTP-bound active $G\alpha_{13}$ [Kashef et al., 2005]. Therefore, it is likely that the JLP-$G\alpha_{13}$ complex observed in RA treated cells involves the GTP-bound $G\alpha_{13}$. It is possible that JLP mediates the stimulation of JNK by retinoids in other systems as well. Although we have not investigated the role of JLP in RA-mediated JNK activation in diverse system, since it is a ubiquitously expressed JNK scaffolding protein, it could potentially play a role in other systems as well. Our results presented here show that the expressions of $G\alpha_{13}$ and JLP are upregulated in RA-treated P19 cells. The novel observation that JLP levels are increased upon RA-treatment is highly significant. Although we have not tested whether the upregulation of JLP is via translational or transcriptional control mechanism(s), the findings that siJLP silences the expression of JLP (Fig. 4B) suggests that the RA-induced expression of JLP is, at least partly, mediated by transcriptional control mechanism(s). The observation that the silencing of JLP using siRNA to JLP results in the inhibition of endodermal differentiation of P19 cells by 50% further emphasizes the critical need for this molecule in mediating primitive endodermal differentiation. Further studies are being carried out to identify novel JLP interacting partners required in the RA-JLP-differentiation pathway.

To summarize, we have demonstrated here, for the first time, a critical role of JLP in endodermal differentiation and the novel interaction between JLP and $G\alpha_{13}$ in P19 cells stimulated by RA. Considering that the activation of JNK is involved in several distinct cellular responses, including cell proliferation; it is possible that JLP plays a multi-faceted role in a cellular and context-specific manner. The recent finding that JLP can also interact with the kinesin motor protein, which plays a role in the proper cellular localization of signaling complexes [Nguyen et al., 2005], suggests the interesting possibility that JLP can regulate G protein mediated signaling complexes in a spatio-temporal manner.

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