Interleukin-11 Induces Complex Formation of Grb2, Fyn, and JAK2 in 3T3L1 Cells*

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Previous studies suggested that interleukin-11 (IL-11) induces the activation of mitogen-activated protein kinase (MAPK) in mouse 3T3L1 cells. However, the mechanisms by which IL-11 activates MAPK remain elusive. Our present results show that IL-11 promotes the formation of the active GTP-bound form of Ras, suggesting that IL-11 actions may be transduced in part through the Ras/MAPK signaling pathway. By immunoblotting and immunoprecipitation, we further demonstrate the association of tyrosine phosphoproteins with Grb2, an adaptor protein serving as a key intermediate for Ras activation. These phosphotyrosine-containing proteins have been subsequently identified to be JAK2, Fyn, and Syp. JAK2 and Fyn are transiently associated with Grb2 upon stimulation with IL-11, suggesting that JAK2 and Fyn may be involved in transducing signals from the IL-11 receptor-glycoprotein 130 to the Ras system through Grb2. Taken together, these results suggest that IL-11-induced interactions of JAK2, Fyn, and Grb2 may not only provide a novel mechanism for the activation of the Ras/MAPK system but also indicate cross-talk among diverse signaling pathways.

IL-11 is a stromal fibroblast-derived cytokine (1, 2) that plays essential roles in a variety of biological systems. Previous studies have suggested that IL-11 depends on gp130 to transduce its signals into cells (3). gp130 is an IL-6 receptor-associated signal transducer and is shared by receptor complexes for leukemia inhibitor factor, oncostatin M, ciliary neurotrophic factor, and IL-11 (3). It has been reported that gp130 associates with Janus kinase 2 (JAK2) tyrosine kinase, which is tyrosine-phosphorylated and activated upon IL-11 stimulation (7). MAPK has been shown to be a key target of Ras (8), a crucial downstream signaling partner for growth factor receptor tyrosine kinases (9, 10). Thus, it is possible that Ras may also be involved in IL-11 signal transduction.

Growth factor receptor binding protein 2 (Grb2) is an adaptor protein containing a Src homology type 2 (SH2) domain flanked by two Src homology type 3 (SH3) domains (9–11). Through its SH3 domains, Grb2 constitutively binds a Ras guanine nucleotide-releasing factor (GNRF) like Sos or Cdc25, which activates Ras by replacing Ras-bound GDP with GTP. In the absence of cellular activation, the Grb2-GNRF complex is located in the cytoplasm. Upon activation by growth factors, the SH2 domain of Grb2 binds activated tyrosine-phosphorylated receptors or receptor-associated tyrosine phosphoproteins, thus bringing GNRF to the plasma membrane where Ras is located (9–11). Through its adaptor-like function, Grb2 plays a key role in linking cell receptor tyrosine kinases and associated tyrosine phosphoproteins with the Ras pathway.

The interactions of Grb2 with receptor tyrosine kinases have been studied in the epidermal growth factor receptor (EGFR) and the insulin receptor (IR) systems (12, 13). Ligand binding leads to EGFR autophosphorylation on multiple tyrosine residues. Subsequently, the SH2 domain of Grb2 becomes attached to tyrosine residues of the EGFR, thus relocating Sos to the plasma membrane. In contrast to the EGFR, stimulation of the IR involves two additional proteins, Shc and insulin receptor substrate-1. Upon stimulation with insulin, Shc and insulin receptor substrate-1 become tyrosine-phosphorylated and bind to Grb2 in order to recruit the Grb2-Sos complex to the cell membrane.

In marked contrast to receptor tyrosine kinases like EGFR and IR, the IL-11 signal transducer gp130 has no kinase domains (3) and needs to activate membrane-associated non-receptor tyrosine kinases for mediating its effects. It is therefore interesting to elucidate which non-receptor tyrosine kinases are recruited by IL-11 to interact with Grb2, hence transducing signals from the activated IL-11 receptor-gp130 complex to the Ras/MAPK pathway.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human IL-11 (specific activity, 2.5 × 10^6 units/mg) was generously provided by Genetics Institute (Cambridge, MA). Anti-phosphotyrosine monoclonal antibody (4G10), and affinity-purified anti-Grb2 and anti-Fyn antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). Affinity-purified anti-Grb2 and anti-Fyn antibodies, together with anti-Fyn monoclonal antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Ras monoclonal antibody (Y13–259) was from Oncogene Science, Inc. (Cambridge, MA). Anti-Syp was a gift from Dr. Gen-Sheng Feng (Indiana University School of Medicine). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were from Amersham Corp., and horseradish peroxidase-conjugated anti-rat IgG was from Zymed Laboratories, Inc. (San Francisco, CA).

Treatment of 3T3-L1 Cells with IL-11, Immunoprecipitation, and Immunoblotting—3T3-L1 mouse preadipocytes were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. Confluent cells were stimulated without or with 500 ng/ml IL-11 at 37 °C as indicated under "Results and Discussion." 500 ng/ml has been
determined to be the optimum concentration of IL-11 on 3T3L1 cells from our previous studies (7). Reactions were stopped by placing dishes of attached cells on ice and adding ice-cold phosphate-buffered saline containing 2 mM sodium orthovanadate to the cells. Cells were scraped off the plate, washed in ice-cold phosphate-buffered saline, and then centrifuged at 500 × g for 5 min at 4°C. The cells were then lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM phenyl-methylsulfonyl fluoride, 1 mM NaF, 1 mM sodium orthovanadate, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). Solubilized proteins were collected after centrifugation (13,000 × g for 10 min at 4°C) and used for immunoprecipitations with the appropriate antibodies for 4-h incubation at 4°C with rotation. The immune complexes were further incubated with protein A-agarose beads (Upstate Biotechnology, Inc., Lake Placid, NY) for 1 h at 4°C with rotation and then washed five times with modified RIPA buffer. The immunoprecipitates were separated by 12 or 7.5% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidine difluoride membranes. The membranes were then immunoblotted with the appropriate antibodies, and antibody-bound proteins were visualized using horseradish peroxidase-conjugated anti-mouse, anti-rabbit, or anti-rat IgG and ECL (Amer sham Corp.) according to the manufacturer's specifications.

In Vitro Binding Assay— GST-Raf (amino acids 51–149) fusion protein and GST alone as a control were freshly prepared for in vitro binding experiments as described (14). 3T3L1 cells were treated in the absence or presence of 500 ng/ml IL-11 at 37°C as indicated under “Results and Discussion.” For in vitro binding studies, glutathione-Sepharose beads (Pharmacia Biotech Inc.) with bound fusion proteins (approximately 5 μg of fusion protein/binding reaction) were incubated with cell lysates in digitonin lysis buffer (1% digitonin, 150 mM NaCl, 2 mM EDTA, 50 mM Tris, pH 8.0, 2 mM sodium orthovanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) at 4°C for 12–14 h. The beads were washed three times with digitonin lysis buffer before the bound proteins were separated by SDS-PAGE and immunoblotted with anti-Ras.

**RESULTS AND DISCUSSION**

**IL-11 Induces the GTP-Ras Formation in 3T3L1 Cells**—In order to test whether Ras was activated by IL-11, the GST-Raf in vitro binding assay was performed using lysates from unstimulated and IL-11-treated 3T3L1 cells. It has recently been shown that Ras protein interacts directly with GST-Raf in vitro, and the binding of Ras and GST-Raf is dependent on the activation of Ras loaded with GTP rather than GDP (14). Therefore, the in vitro binding assay of Ras and GST-Raf is suggested to be a useful approach to verify the activation of Ras (14). In this study, we demonstrated that IL-11 induces complex formation between Ras and GST-Raf (amino acids 51–149) in vitro (Fig. 1). 3T3L1 cells were treated without or with 500 ng/ml IL-11 for 3 min and lysed with digitonin-containing lysis buffer. Cell lysates were immunoprecipitated with anti-Ras (Y13–259) or incubated with GST-Raf fusion protein or GST alone (as a negative control) immobilized to glutathione-Sepharose. The samples were washed, and bound proteins were separated by SDS-PAGE and immunoblotted with anti-Ras (Y13–259). As shown in Fig. 1, 3T3L1 cells with IL-11 had no effect on the protein level of Ras as was determined by immunoprecipitation with anti-Ras. Ras complexed with GST-Raf fusion proteins in the lysates from IL-11-stimulated 3T3L1 cells, whereas no GST-Raf-associated Ras was detected from unstimulated 3T3L1 cells. These results in conjunction with our previous studies (7) indicate that IL-11 signaling is at least in part mediated through the Ras/MAPK pathway.

Phosphotyrosine-containing Proteins Associate with Grb2 in 3T3L1 Cells—The adaptor protein Grb2 is a key intermediate to facilitate protein-protein interactions and to transduce signals from ligand-activated membrane receptors to the Ras/ MAPK pathway. To analyze the pattern of phosphotyrosine-containing proteins associated with Grb2 following IL-11 stimulation, lysates in modified RIPA buffer from IL-11-treated or untreated 3T3L1 cells were immunoprecipitated with anti-Grb2 antibody, and the precipitated proteins were analyzed by immunoblotting with anti-phosphotyrosine mAbs (4G10). As shown in Fig. 2, Grb2 constitutively associated with a tyrosine phosphoprotein with a molecular mass around 75 kDa, and IL-11 stimulation did not seem to affect their association. However, IL-11 induced two more phosphotyrosine-containing proteins of 130 and 60 kDa to complex with Grb2. After IL-11 treatment, the association of these two phosphotyrosine-containing proteins with Grb2 occurred at 1 min, could still be observed at 3 min, and completely disappeared at 7 min. 1 and 7 min are selected to be the two time points of IL-11 treatment for subsequent experiments. The same immunoprecipitates were rebotted with anti-Grb2 to verify the same amount of Grb2 in each immunoprecipitate. The 75-kDa tyrosine phosphoprotein was further identified as Syp (data not shown). Syp is a newly discovered protein-tyrosine phosphatase (15), and the function of its association with Grb2 in IL-11-triggered signal transduction requires further investigation.

**Grb2 Associates with Tyrosine-phosphorylated Fyn**—In response to IL-11—To identify the 60-kDa Grb2-associated tyrosine-phosphorylated protein, anti-Grb2 immunoprecipitates were immunoblotted with anti-Shc. Shc has been shown to complex with Grb2 and activate the Ras signaling pathway upon stimulation with several cytokines (16, 17). However, in our experiments Shc was not detected in anti-Grb2 immunoprecipitates from both unstimulated and IL-11-treated 3T3-L1 cells (data not shown). We therefore examined whether Src family tyrosine kinases, including Fyn, Yes, and Src, are possible candidates for this 60-kDa tyrosine-phosphorylated protein. Fyn, Yes, and Src have been determined from our previous studies to be the three Src family kinases expressed in 3T3L1 cells (data not shown). As shown in Fig. 3A, Fyn was not present in anti-Grb2 immunoprecipitates from unstimulated…
myristoylation of p56
GTP-activating protein required the enzymatic activation and cells were stimulated by IL-11 for 1 min, as shown in Fig. 3
the presence of Grb2 in the anti-Fyn immunoprecipitates after reciprocal immunoprecipitation and immunoblotting confirmed stimulated or IL-11-treated 3T3L1 cells (data not shown). The detected in anti-Grb2 immunoprecipitates from either un-
cells were exposed to IL-11 for 7 min. Src and Yes could not be
were stimulated with IL-11 for 1 min and disappeared after cells were treated by IL-11 for 1 min (Fig. 4A). JAK2 was not detected in anti-Grb2 immunoprecipitates from either unstimulated or IL-11-treated 3T3L1 cells (data not shown). The reciprocal immunoprecipitation and immunoblotting confirmed the presence of Grb2 in the anti-Fyn immunoprecipitates after cells were stimulated by IL-11 for 1 min, as shown in Fig. 3B. Fyn has a basal level of tyrosine phosphorylation in the unstimulated 3T3-L1 cells. However, tyrosine phosphorylation of Fyn was enhanced transiently following IL-11 stimulation (Fig. 3B). Fyn and other Src family kinases have been reported to be associated with signaling molecules, including phospholipase C-γ2 and PI3-kinase, which phosphorylates the Asp-3 position of phosphatidylinositol in various cytokine signal transduction mechanisms (18–20). It has also been reported that Src family kinases are involved in the regulation of Ras activity. The Ras GTP-activating protein, an important regulator of the Ras function, was found in complexes with v-Src and c-Src and was phosphorylated by activated Src kinase in v-Src- and c-Src-transformed cells (21). In NIH3T3 fibroblasts expressing oncogenic and transformation-defective variants of the Src family kinase p56\(^{ck}\), tyrosine phosphorylation and activation of Ras GTP-activating protein required the enzymatic activation and myristoylation of p56\(^{ck}\) (22). Recently, the p120\(^{ck}\) protooncoprotein has been shown to form a complex with Fyn, Grb2, and PI3-kinase (23). The association of p120\(^{ck}\) with Fyn and PI3-kinase was markedly increased by T cell activation. In contrast, the association of p120\(^{ck}\) with Grb2 did not change upon T cell activation. Our present results further suggest that Fyn can be recruited to interact with Grb2 following IL-11 stimulation, thus taking a part in transducing signals from the IL-11 receptor-gp130 complex to the Ras pathway.

IL-11 induces tyrosine-phosphorylated JAK2 to Complex with Grb2 and Fyn as well as gp130. 3T3L1 cells were treated without or with IL-11 (500 ng/ml) for the indicated times. Cell lysates were immunoprecipitated with anti-Grb2 (A), anti-J AK2 (B), or anti-gp130 (C). The immunoprecipitates (IP) were resolved by SDS-PAGE and sequentially immunoblotted with anti-J AK2 and anti-Grb2 (A), anti-Grb2, anti-Fyn (mAb), anti-phosphotyrosine mAb (4G10), and anti-J AK2 (B), or anti-J AK2 and anti-gp130 (C).

Fig. 4. IL-11 induces tyrosine-phosphorylated J AK2 to complex with Grb2 and Fyn as well as gp130.

A

B

C

Fig. 3. Tyrosine-phosphorylated Fyn associates with Grb2 in response to IL-11. 3T3L1 cells were treated without or with IL-11 (500 ng/ml) for the indicated times. Cell lysates were immunoprecipitated with either anti-Grb2 (A) or anti-Fyn (B). The immunoprecipitates (IP) were resolved by SDS-PAGE and sequentially immunoblotted with anti-Fyn and anti-Grb2 (A) or anti-Grb2, anti-phosphotyrosine mAb (4G10), and anti-Fyn (B).
Fyn after IL-11 treatment (Fig. 4B). It has been theorized that protein-protein interactions may cause a conformational change in the Src family kinases, thus leading to the activation of the Src family kinases (27). J AK2 has also been found to form a complex containing Src and PI3-kinase in response to IL-3 in NIH3T3 cells (28). It is therefore possible that J AK2 may associate with and hence activate Fyn after IL-11 stimulation.

In summary, our data demonstrate that IL-11 stimulation induces a novel complex formation among J AK2, Fyn, and Grb2. The complex may serve to propagate signals originating from the IL-11 receptor-gp130 or alter the subcellular location of key signaling molecules such as Grb2-bound Ras GNRF, Grb2-complexed GNRFs identified so far are Sos1 (29), Sos2 (29), Cdc25 (30), and C3G (31). Our preliminary experiments failed to demonstrate the association between mSos1 and J AK2 or Fyn (data not shown). Further investigations will be required to identify the Grb2-bound GNRF, which is involved in IL-11-induced Ras activation. In addition to providing a novel mechanism that activates the Ras/MAPK signaling pathway, IL-11-induced Ras activation. In addition to providing a novel mechanism that activates the Ras/MAPK signaling pathway, IL-11-induced complex formation of Grb2, J AK2, and Fyn may also represent the interactions among JAK/Stat, Ras/MAPK, and other Src family kinase-related signaling pathways. These interactions may result in diverse signaling pathways functioning in concert to dictate different cellular responses.

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