Communication

Activation of a CrkL-Stat5 Signaling Complex by Type I Interferons*

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Type I interferons (IFNα and IFNβ) transduce signals by inducing tyrosine phosphorylation of JakS and Stats, as well as the CrkL adapter, an SH2/SH3-containing protein which provides a link to downstream pathways that mediate growth inhibition. We report that Stat5 interacts constitutively with the IFN receptor-associated Tyk-2 kinase, and during IFN stimulation its tyrosine-phosphorylated form acts as a docking site for the SH2 domain of CrkL. CrkL and Stat5 then form a complex that translocates to the nucleus. This IFN-inducible CrkL-Stat5 complex binds in vitro to the TTCTAGGAATTCAAATC palindromic sequence found in the promoters of IFN-stimulated genes. Thus, during activation of the Type I IFN receptor, CrkL functions as a nuclear adapter protein and, in association with Stat5, regulates gene transcription through DNA binding.

Type I interferons (IFNα, IFNβ, and IFNω) are pleiotropic cytokines that exhibit multiple biological effects including antiviral and growth-inhibitory activities (1, 2). Following engagement of the Type I IFN receptor by IFNω or IFNβ, two kinases of the Janus family, Tyk-2 and Jak-1, are activated and phosphorylate the Stat proteins: Stat1, Stat2, Stat3, Stat4, and Stat5 (3, 4). Activated Stat proteins form distinct signaling complexes to regulate gene transcription. Stat1 and Stat2 form a heterodimer that associates with a member of the IFN regulatory factor family, p48, resulting in the formation of the mature ISGF3 complex that translocates to the nucleus to initiate gene transcription by binding to interferon-stimulated response elements (3, 4). Stat1 and Stat3 homo- and heterodimers and homodimers of Stat4, Stat5a, and Stat5b bind a palindromic sequence found in the promoters of IFN-stimulated genes (4, 5).

In addition to the Stat pathway, other signaling pathways are activated downstream of JakS in IFNω signaling. These include the insulin receptor substrate (IRS) pathway that regulates activation of the phosphatidylinositol 3'-kinase (6–8) and the CrkL pathway that links the functional Type I IFN receptor complex to the growth-inhibitory C3G/Rap-1 cascade (9). In the present study we determined whether the CrkL pathway functions in coordination with the Stat pathway. Our data demonstrate that Stat5 is constitutively associated with the Tyk-2 kinase, and its IFN-phosphorylated form provides a docking site for the SH2 domain of CrkL. The resulting CrkL-Stat5 complex translocates to the nucleus to regulate gene transcription via GAS elements. Viewed together, these findings provide evidence for a novel function of CrkL as a nuclear adapter protein.

Experimental Procedures

Cells and Reagents—The Daudi and KG1 human cell lines were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Inc.) and antibiotics. Human recombinant IFNω2 was provided by Hoffmann-La Roche. Human recombinant IFNα-consensus (IFNCon1) was provided by Amgen Inc. Human recombinant IFNβ was provided by Biogen Inc. (Cambridge, MA). The anti-CrkL and anti-Stat5b polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The production of the pGEX-CrkLSH2 construct has been described previously (10).

Immunoprecipitations, Immunoblotting, and Glutathione S-Transferase Binding Studies—Cells were stimulated with 104 units/ml of the indicated interferons as described previously (9). After stimulation, the cells were lysed in phosphorylation lysis buffer, and immunoprecipitations and immunoblotting using the ECL method were performed as described previously (9). Production of glutathione S-transferase fusion proteins and binding experiments using lysates from IFNω-untreated or -treated cells were performed as described previously (9, 11).

Genomic DNA Affinity Chromatography and Mobility Shift Assays—Preparation of nuclear extracts, genomic DNA affinity chromatography, and mobility shift assays were performed essentially as described previously (12). A double-stranded oligodeoxynucleotide specific for Stat5 binding (AGATTCTAGGAAATTTCAAATC), derived from the β-casein promoter, was synthesized and used in gel shift assays.

Results and Discussion

We sought to identify the tyrosine kinase that regulates IFNω-induced activation of Stat5 and the mechanisms by which the protein is activated and binds DNA. When lysates from IFNω-stimulated KG1 myeloid cells were immunoprecipitated with an anti-Tyk-2 antibody and immunoblotted with antiphosphotyrosine, we noted that a tyrosyl phosphoprotein migrates as a doublet at 96/94 kDa was complexed with Tyk-2 (Fig. 1A). This protein corresponded to Stat5, as determined by immunoblotting with a specific anti-Stat5 antibody (Fig. 1B). The interaction of Stat5 with Tyk-2 was present prior to IFNω treatment and increased further after IFNω stimulation, suggesting that Stat5 interacts constitutively with Tyk-2 and thus may provide a link between this kinase and downstream signaling elements. Similarly, a constitutive interaction of Stat5 with Tyk-2 was seen in studies with the IFNω-sensitive Daudi lymphoblastoid cell line (Fig. 1, C and D).

In previous studies, we have demonstrated that the adapter protein CrkL interacts in an IFNω-dependent manner with Tyk-2 and is tyrosine-phosphorylated during IFNω stimulation.
tion, providing a link between the Type I IFN receptor and the C3G-Rap1 growth-inhibitory pathway (9). Accordingly, we examined whether CrkL associates with Stat5 during IFNa or IFNb stimulation. In time course studies, Daudi cells were left untreated or treated for 10 or 20 min with IFNa or IFNb. Cell lysates were prepared, immunoprecipitated with an anti-CrkL antibody, and then immunoblotted with an anti-Stat5 antibody. Stat5 was detected in association with CrkL after IFNa stimulation, establishing that this member of the Stat family of transcription factors is involved in Type I IFN signaling. Since IFNa treatment activates Type I IFN-dependent genes, we tested whether this association is dependent on IFNa stimulation. Indeed, Daudi cells were incubated in the presence or absence of IFNa, and Stat5 was shown to associate with CrkL in an IFNa-dependent manner (Fig. 2, A and B). In contrast, there was no Stat5 present in anti-CrkL immunoprecipitates from IFNb-treated cells, suggesting that the IFNa treatment is required for the association of CrkL with Stat5. Next, we examined whether the presence of IFNa is required for the association of CrkL with Stat5. Daudi cells were incubated in the presence or absence of IFNa. Cell lysates were immunoprecipitated with anti-Tyk-2 antibody, and then immunoblotted with an anti-Stat5 antibody. Cell lysates were immunoprecipitated with anti-Tyk-2 antiserum or nonimmune RlgG, and proteins were analyzed by SDS-PAGE and immunoblotted with an anti-Stat5 antibody. The blot shown in C was stripped and reprobed with a monoclonal antibody against Tyk-2 (Transduction Laboratories).

Fig. 1. Interaction of Stat5a and Stat5b with the Type I IFN receptor-associated Tyk-2 tyrosine kinase. A, KG-1 cells were incubated with IFNa for the indicated times at 37 °C. Cell lysates were immunoprecipitated with an anti-Tyk-2 antiserum or nonimmune rabbit serum (NRS), and proteins were analyzed by SDS-PAGE and immunoblotted with an antiphosphotyrosine monoclonal antibody (4G-10, Upstate Biotechnology). B, the blot shown in A was stripped and reprobed with an antibody against Stat5b that recognizes both forms of Stat5 (Santa Cruz Biotechnology). C, Daudi cells were incubated at 37 °C for the indicated times in the presence or absence of IFNa. Cell lysates were immunoprecipitated with anti-Tyk-2 antiserum or nonimmune RlgG, and proteins were analyzed by SDS-PAGE and immunoblotted with an anti-Stat5 antibody. D, the blot shown in C was stripped and reprobed with a monoclonal antibody against Tyk-2 (Transduction Laboratories).

Fig. 2. IFNa and IFNb induce the association of CrkL with Stat5 in intact cells. A, Daudi cells were treated for the indicated times with IFNa or IFNb. Cell lysates were first precleared with nonimmune RlgG and after immunoprecipitation with the indicated antibodies, analyzed by SDS-PAGE and immunoblotted with an anti-Stat5b antibody. B, the blot shown in A was stripped and reprobed with the anti-CrkL antibody to demonstrate equal loading.

Fig. 3. The IFNa-induced tyrosine-phosphorylated form of Stat5 functions as a docking site for the SH2 domain of CrkL. A, Daudi cells were incubated in the presence or absence of IFNa for 20 min. Cell lysates were immunoprecipitated with an antibody against Stat5b and immunoblotted with antiphosphotyrosine. B, the blot shown in A was stripped and reprobed with the anti-Stat5b antibody. C, Daudi cells were incubated at 37 °C for 30 min in the presence or absence of IFNa. Cell lysates were bound to a glutathione S-transferase fusion protein encoding the SH2 domain of CrkL (GST-CkLSH2) or GST alone used as control. Bound proteins were analyzed by SDS-PAGE and immunoblotted with an antibody against Stat5b. D, KG-1 cells were incubated at 37 °C for 30 min in the presence or absence of IFNa. Cell lysates were bound to glutathione S-transferase fusion protein encoding the SH2 domain of CrkL (GST-CkLSH2) or GST alone used as control. Bound proteins were analyzed by SDS-PAGE and immunoblotted with an antibody against Stat5b.

Further analyses of these Type I IFN-induced nuclear extracts by gel shift assays, employing an oligonucleotide specific for the interferon-gamma-responsive element of the C3G-Rap1 growth-inhibitory pathway (9). Specifically, nuclear extracts from Daudi cells treated with IFNa or IFNb were subjected to gel retardation analysis (GAD), and the DNA-bound fraction was resolved by SDS-PAGE and immunoblotted for C3G (Fig. 4A). C3G bound DNA in an IFNa- or IFNb-dependent manner (Fig. 4A), and its DNA-bound form migrated at approximately 140 kDa, strongly suggesting that such DNA binding occurs in a complex with Stat5. Furthermore, as C3G was detectable in immunoblots of the nuclear extracts only after Type I IFN treatment, these data suggested that the protein translocates to the nucleus in a Type I IFN-dependent manner. Similarly, when the IFN-induced DNA-bound fractions collected following GAD were immunoblotted with Stat5, we noticed that Stat5 was detectable in the same complex with C3G (Fig. 4B), strongly suggesting that it forms a DNA-binding complex in association with CrkL.

2 S. Uddin and L. C. Platanias, unpublished observations.
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for Stat5 binding derived from the β-casein promoter, identified the presence of IFNa- or IFNβ-inducible DNA-binding complexes, whose mobilities were affected by inclusion of anti-CrkL antibodies (Fig. 5A) but not control IgG (Fig. 5A and data not shown). The presence of Stat5 in these CrkL-containing complexes in the electrophoretic mobility shift assay was confirmed by immunoblotting with antibodies to Stat5 (Fig. 5B). Thus, CrkL forms DNA-binding complexes in association with Stat5, strongly suggesting that it is involved in the regulation of Type I IFN-dependent gene expression.

Our data provide strong evidence that CrkL, in cooperation with Stat5, binds DNA, and this complex likely functions as a transcription factor in IFNa/β-induced signaling. Indeed, we have observed this IFN-induced CrkL-Stat5 complex in other IFN-sensitive cell lines, namely human glial T98G and human osteosarcoma U2OS cells (data not shown). Such a role for CrkL was unexpected and raises the possibility that other related proteins, e.g., CrkII, Grb-2, may function as transcriptional activators in other signaling cascades. The members of this family of proteins have been previously shown to function as adapters, providing a link between receptor tyrosine kinases or their substrates and guanine exchange factors. Until now, there has been no evidence of their exhibiting DNA binding activity. CrkL has been shown to interact primarily with C3G (9, 13–15), which acts as a guanine exchange factor for Rap-1 (16), a small G protein that antagonizes Ras and has tumor suppressor activity (17–19). Regulation of Rap-1 activation by the CrkL-C3G complex appears to be critical for inhibition of T-cell proliferation and induction of anergy (20). In addition, recent studies have demonstrated that CrkL interacts with a newly cloned member of the IRS family of proteins, IRS-4, in an IGF-1-dependent manner and that it has oncogenic potential when overexpressed in cell lines (21).

The current report implicates Stat5 in the engagement of CrkL in IFN signaling, as shown by the requirement of Stat5 as a docking site for the SH2 domain of CrkL. Most importantly, for the first time these data demonstrate that a Stat protein can act as a docking protein for the SH2 domain of a non-Stat protein to form a DNA-binding complex with it. Although, in the case of CrkL, this function appears to be specific for Stat5, it is likely that other Stats will be found to function in a similar manner in other systems. Recent reports have suggested that Stat5 is involved in IFNα signaling in myeloid cell lines and HeLa cells (5), and its activation has been observed in response to differentiation and growth arrest signals (22, 23). Our results strongly suggest that such functions for Stat5 require its interaction with and formation of a signaling complex with CrkL.

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