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Preassociation of STAT1 with STAT2 and STAT3 in Separate Signalling Complexes Prior to Cytokine Stimulation*

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Louis F. Stancato‡, Michael David§, Christin Carter-Su¶, Andrew C. Larner§, and William B. Pratt‡‖

From the Departments of ‡Pharmacology and ¶Physiology, University of Michigan Medical School, Ann Arbor, Michigan 48109 and the §Division of Cytokine Biology, Food and Drug Administration, Bethesda, Maryland 20892

A variety of cytokines and growth factors act through an induction of gene expression mediated by a family of latent transcription factors called STAT (signal transducers and activators of transcription) proteins. Ligand-induced tyrosine phosphorylation of the STATs promotes their homodimer and heterodimer formation and subsequent nuclear translocation. We demonstrate here that STAT protein heterocomplexes exist prior to cytokine treatment. When unstimulated HeLa cells are ruptured in hypotonic buffer without salt or detergent, immunoadsorption of either STAT1 or STAT2 from the resulting cytosol yields coimmunoadsorption of the other STAT protein. Similarly, STAT1-STAT3 heterocomplexes are coimmunoadsorbed from hypotonic cytosol. STAT1 and STAT2 or STAT1 and STAT3 translated in reticulocyte lysate spontaneously form heterocomplexes when the translation lysates are mixed at 0 °C. Our data suggest that interferon-α/β-induced tyrosine phosphorylation increases the stability of a preexisting, latent, STAT1-STAT2 signaling complex. Newly translated STAT1 binds in equilibrium fashion to STAT2 and STAT3, but we show that STAT2 and STAT3 exist in separate heterocomplexes with STAT1, consistent with a model in which STAT1 contains a common binding site for other STAT proteins.

It is now appreciated that a large number of cytokines, growth factors, and hormones act through a sequence of steps that includes binding to receptors in the cytokine receptor superfamily followed by activation of the JAK (Janus kinases) family of tyrosine kinases, which results in activation of STAT (signal transducers and activators of transcription) proteins that subsequently migrate to the nucleus where they initiate specific gene transcription (see Refs. 1 and 2 for review). The overall signaling pathway is utilized by the interferons (IFNs)[1] and a variety of other cytokines, including the interleukins, some colony stimulating factors (granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor), leukemia inhibitory factor (LIF), oncostatin M, and erythropoietin, as well as the peptide hormones prolactin and growth hormone. The JAK family of kinases includes JAK1, JAK2, JAK3, and Tyk2, and, to date, six STAT proteins have been identified.

One of the most studied cytokine signaling systems is interferon-mediated gene activation. Here it is known that interaction of IFN-α or IFN-β with the IFN-α/β receptor activates JAK1 and Tyk2 with resulting tyrosine phosphorylation of the 91-kDa STAT1 and the 113-kDa STAT2 proteins, which are then thought to form a STAT heterodimer that associates with a 48-kDa DNA-binding protein. This multiprotein unit is called the interferon-stimulated gene factor 3, and it appears to be the primary positive regulator of interferon-stimulated response element-controlled genes (3–5). Thus, the STAT proteins are essentially latent transcription factors residing outside of the nucleus (6), and *in vivo* activation is associated with interferon-stimulated gene factor 3 translocation to the nucleus (5, 7).

We started the work we report herein asking whether we could use the gentle techniques of cell rupture and immunoadsorption in hypotonic buffer that we have used to study steroid receptor and protein kinase heterocomplexes (8) to detect possible complexes between STAT proteins and hsp90. We were not able to detect any association of STAT1, -2, or -3 with hsp90, but we serendipitously made some fundamental observations regarding the formation of STAT protein complexes themselves. We found that rupture of HeLa cells in a hypotonic buffer followed by immunoadsorption of STAT1 yields coimmunoadsorption of STAT2 and *vice versa*. This suggests that in untreated cells the two proteins exist together in a complex but that the association is weak, as the complex is not observed after cell rupture in buffer containing 1% Triton X-100 and 150 mM NaCl. After stimulation with IFN-α or IFN-β, however, the STAT1-STAT2 complex survives the "harsh" rupture conditions with detergent and salt. After stimulation by IFN-γ, which is thought to induce gene expression through an interaction of tyrosine-phosphorylated STAT1 homodimers with γ activation sequences (9), no STAT1-STAT2 complex is seen under the harsh rupture conditions.

Our data point to a revision of the standard model where it has been assumed that signaling by IFN-α/β receptors leads to STAT protein phosphorylation with subsequent heterocomplex formation. Rather, tyrosine phosphorylation seems to increase the stability of a preexisting, latent signaling complex. In the absence of cytokine stimulation, we have found that STAT1 and STAT2 associate with each other in an equilibrium fashion, as do STAT1 and STAT3, but STAT2 and STAT3 exist only in separate heterocomplexes with STAT1. This may imply that STAT1 contains a common binding site for other STAT proteins.

EXPERIMENTAL PROCEDURES

Materials

^{125}I-Conjugated goat anti-mouse and anti-rabbit IgGs, and ^{35}S-methionine (translation grade) were obtained from DuPont NEN. Protein

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‖ To whom correspondence should be addressed: Dept. of Pharmacology, 1301 Medical Science Research Bldg. III, University of Michigan Medical School, Ann Arbor, MI 48109-0632. Tel.: 313-764-5414; Fax: 313-763-4450.

[1] The abbreviations used are: IFN, interferon; LIF, leukemia inhibitory factor; hsp, heat shock protein; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino} ethanesulfonic acid (systematic).

A-Sepharose and goat anti-mouse and anti-rabbit IgG horseradish peroxidase conjugates were from Sigma. The rabbit antisera against STAT1 and STAT2 have been described previously (10). The rabbit antiserum against STAT3 was raised against a peptide identical to the 38 C-terminal amino acids of STAT3. The anti-STAT3 monoclonal IgG antibody and the monoclonal anti-phosphotyrosine antibody (4G10) were from Transduction Laboratories. Leukemia inhibitory factor was from Promega. Recombinant human IFN-α-2a was from Hoffman La-Roche, and recombinant human IFN-β was from Chiron Inc. (Emeryville, CA). Recombinant human IFN-γ was from Genentech. *In vitro* coupled transcription/translation kits were from Promega.

Methods

Cell Culture and Fractionation—HeLa cells and 3T3-F442A mouse fibroblasts were grown in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. Where indicated, HeLa cells were incubated with 1000 units/ml IFN-α or IFN-β, or 10 ng/ml of IFN-γ, for 20 min at 37 °C. 3T3-F442A fibroblasts were incubated with 25 ng/ml of LIF for 20 min at 37 °C. Cells were harvested by scraping into Earle's balanced saline followed by a second wash and centrifugation at 500 × *g*. The washed cells were suspended in 1 volume of HE buffer (10 mM Hepes, pH 7.35, 1 mM EDTA), which we call "hypotonic buffer," or in HE + 1% Triton X-100 + 150 mM sodium chloride ("harsh buffer") and ruptured by Dounce homogenization. Homogenates were then centrifuged for 10 min at 12,000 × *g* at 4 °C, with the supernatant from this step being the "cytosol" from which the STAT proteins were immunoadsorbed.

STAT Protein Immunoadsorption—STAT proteins were immunoadsorbed from 200-μl aliquots of cytosol by first incubating with 5 μl of antiserum for 1 h on ice, followed by incubation with 35 μl of a 20% slurry of protein A-Sepharose for 1 h on ice. Immunopellets were then washed 3 times with 1 ml of TEG buffer (10 mM TES, 4 mM EDTA, 10% glycerol, 50 mM NaCl, pH 7.6) at 4 °C.

Western Blotting of STAT Proteins—For assay of STAT-associated proteins, immune pellets were assayed by SDS-polyacrylamide gel electrophoresis and immunoblotting. SDS-polyacrylamide gel electrophoresis was performed in 7% slab gels as described previously (11). Immunoblotting was carried out by transferring proteins from acrylamide slab gels to Immobilon P transfer membranes, followed by incubation for 2 h at room temperature with 0.1% anti-STAT1 or anti-STAT2 rabbit antiserum or with 1 μg/ml anti-STAT3 monoclonal antibody. The immunoblots were then incubated a second time with the appropriate horseradish peroxidase-conjugated counterantibody to visualize the immunoreactive bands and a third time with the appropriate ^{125}I-labeled counter antibody for autoradiography. In most cases, with appropriate cutting of the immunoblot, STAT1, STAT2, and STAT3 can be assayed on a single immunotransfer.

Western Blotting of Tyrosine-phosphorylated STAT Proteins—For assay of tyrosine phosphorylation of STAT proteins, Immobilon P membranes previously probed for STAT proteins and subsequently visualized by immunostaining and autoradiography were first stripped of bound antibody by incubating the membrane for 30 min at 50 °C in stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol). The membranes were then incubated for 2 h at room temperature with 1 μg/ml anti-phosphotyrosine monoclonal antibody. The immunoblots were then incubated a second time with the appropriate horseradish peroxidase-conjugated counterantibody to visualize the immunoreactive bands.

In Vitro Transcription and Translation of STAT Proteins—STAT proteins were translated *in vitro* using the Promega TnT-coupled transcription/translation system in rabbit reticulocyte lysate, which allows for both transcription and translation of a protein to occur in a single step. All STAT proteins were translated using 1–3 μg of their respective plasmids (12–14). The translation reactions (50 μl) were carried out for 1 h at 30 °C. The samples were then placed on ice for 15 min to stop translation. Where indicated, the translation reaction mixtures of individual STAT proteins were mixed together and incubated on ice for 30 min prior to immunoadsorption. STAT proteins were immunoadsorbed from the reticulocyte lysate translation mixtures as described above for cytosols, and the relevant proteins were assayed by Western blotting. The ^{35}S-methionine-labeled STAT proteins were visualized by both autoradiography and Western blotting.

RESULTS

STAT1 and STAT2 Are Preassociated—In the experiment of Fig. 1, HeLa cells were ruptured under two conditions: 1) in a hypotonic buffer (lacking salt or detergent) that has been used to isolate heat shock protein heterocomplexes in which some of the proteins are rather weakly bound (15) or 2) in the same buffer but with the addition of 1% Triton X-100 and 150 mM NaCl. This buffer contains detergent and salt to make it similar to the radioimmune precipitation assay buffer that is often used for cell rupture in studies of STAT protein phosphorylation (*e.g.* Ref. 16). It can be seen that immunoadsorption of hypotonic cytosols (from both untreated and interferon-treated cells) with antiserum to STAT1 yields coadsorption of STAT2 (*lane 2*) and that immunoadsorption of STAT2 yields coimmunoadsorption of some STAT1 (*lane 3*). Immunoadsorption of cytosols prepared from untreated or IFN-γ-treated cells with buffer containing detergent and salt does not result in coimmunoadsorption of the cognate STAT protein (*lanes 4* and *5*, *top row*). However, after treatment of cells with IFN-α or IFN-β, the heterocomplex survives cytosol preparation under harsh conditions (*lanes 4* and *5*, *bottom row*). By immunoblotting with anti-phosphotyrosine antibody, it was shown that STAT2 is phosphorylated after treatment with IFN-α and IFN-β but not after treatment with IFN-γ and that STAT1 was phosphorylated after treatment with all three IFNs (data not shown).

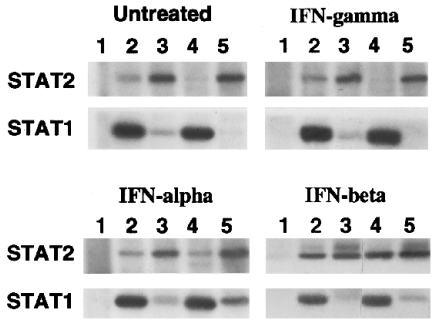

FIG. 1. **STAT1 and STAT2 are associated with each other prior to stimulation with IFN-α or IFN-β.** Untreated HeLa cells or HeLa cells treated with IFN-α, IFN-β, or IFN-γ were assayed for STAT protein complexes. Aliquots (200 μl) of cytosol prepared from hypotonic lysates (*lanes 1–3*) or from detergent/salt lysates (*lanes 4* and *5*) were immunoadsorbed with nonimmune rabbit serum, anti-STAT1, or anti-STAT2. After washing the immunopellets, STAT proteins were resolved by SDS-polyacrylamide gel electrophoresis and Western blotted with anti-STAT1 or anti-STAT2, as designated to the *left*. Under each condition of cell treatment, cytosols were immunoadsorbed with nonimmune serum (*lane 1*), anti-STAT1 (*lane 2*); anti-STAT2 (*lane 3*); anti-STAT1 (*lane 4*), and anti-STAT2 (*lane 5*).

Because STAT1 and STAT2 were coimmunoadsorbed from hypotonic cytosols of untreated cells, we asked if the two proteins would spontaneously associate with each other when mixed together. STAT1 and STAT2 were transcribed and translated in rabbit reticulocyte lysate and then immunoadsorbed with anti-STAT antisera. As shown in Fig. 2 (*lanes 7–9*), when the two proteins were translated in the same mix, immunoadsorption of either STAT1 or STAT2 yielded coimmunoadsorption of the other. It is unlikely that protein folding reactions are required to form the STAT protein heterocomplex because simply mixing two aliquots of reticulocyte lysate containing newly translated STAT1 or STAT2 at 0 °C yields a heterocomplex that can be immunoadsorbed (Fig. 2, *lanes 10* and *11*).

STAT1 and STAT3 Are Preassociated—STAT1 and STAT3 complexes have been detected in lysates of 3T3-F442A cells treated with LIF (17, 18). In the experiment of Fig. 3, untreated 3T3-F442A cells or cells treated with LIF were ruptured in either hypotonic buffer or detergent-containing buffer, and the resulting cytosols were immunoadsorbed with anti-STAT1 or anti-STAT3 antisera. Immunoadsorption of hypotonic cytosol with anti-STAT1 yields coimmunoadsorption of STAT3 (*lane 2*), and immunoadsorption of STAT3 yields coimmunoadsorption of STAT1 (*lane 3*). Rupture of the 3T3 cells in buffer

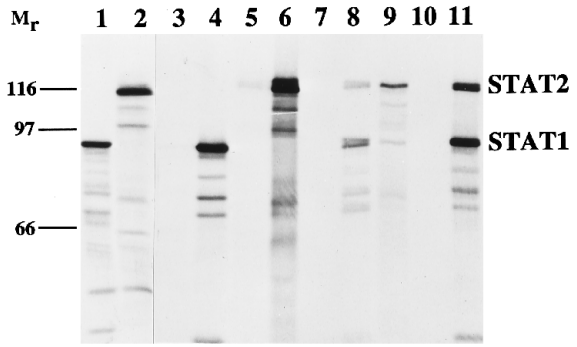

FIG. 2. ***In vitro* translated STAT1 and STAT2 form a complex in rabbit reticulocyte lysate.** STAT1 and STAT2 were transcribed and translated in rabbit reticulocyte lysate. Shown is an autoradiogram of [^{35}S]methionine-labeled proteins in aliquots of reticulocyte lysate translation mixture or in immunoprecipitates prepared from the translation mixture. *Lane 1*, 5 μl of STAT1 translation mixture; *lane 2*, 5 μl of STAT2 translation mix; *lanes 3* and *4*, preimmune and anti-STAT1 immunoadsorption of STAT1 translation mix; *lanes 5* and *6*, nonimmune and anti-STAT2 immunoadsorption of STAT2 translation mix; *lanes 7–9*, nonimmune, anti-STAT1, or anti-STAT2 immunoadsorption from a translation mixture containing both STAT1 and STAT2 cDNAs; *lanes 10* and *11*, separate STAT1 and STAT2 translation mixtures combined on ice and then immunoadsorbed with nonimmune serum (*lane 10*) or anti-STAT1 antiserum (*lane 11*).

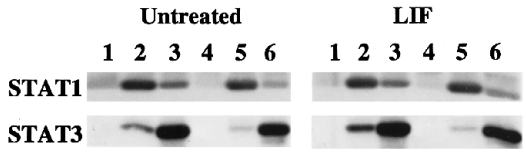

FIG. 3. **STAT1 and STAT3 exist in a preformed complex in 3T3-F442A cell cytosol.** Untreated or LIF-treated 3T3 cells were ruptured in hypotonic buffer (*lanes 1–3*) or buffer with detergent and salt (*lanes 4–6*), and the cytosols were immunoadsorbed with nonimmune serum (*lanes 1* and *4*), anti-STAT1 serum (*lanes 2* and *5*), or anti-STAT3 serum (*lanes 3* and *6*).

containing detergent and salt results in less coadsorption of the cognate STAT protein (*lanes 5* and *6*), and treatment with LIF does not increase heterocomplex recovery after salt treatment. In the experiment of Fig. 3, LIF stimulation of the 3T3 cells led to phosphorylation of both STAT1 and STAT3 as verified by immunoblotting with anti-phosphotyrosine antibody (data not shown).

As shown in Fig. 4 when STAT1 and STAT3 were cotranslated in the same translation mixture, immunoadsorption of STAT3 yielded coimmunoadsorption of STAT1 (*lanes 4* and *5*). Despite the fact that heterocomplexes could be demonstrated in a STAT1/STAT3 cotranslation mix as well as in a STAT1/STAT2 cotranslation mix (*lanes 6* and *7*), no heterocomplex could be demonstrated in STAT2/STAT3 cotranslation (*lanes 8* and *9*).

STAT2 and STAT3 Exist in Separate Heterocomplexes with STAT1—The data of Fig. 5*A* show that STAT2 and STAT3 are not recovered in the same heterocomplex with STAT1. 3T3 cells contain substantial amounts of all three STAT proteins, and it was of interest to ask whether STAT2 and STAT3 were present in the same preformed heterocomplexes with STAT1 in hypotonic lysates. As shown in Fig. 5*A*, immunoadsorption of STAT1 yields coimmunoadsorption of both STAT2 and STAT3 (*lane 3*), but immunoadsorption of STAT3 (*lane 2*) or STAT2 (*lane 4*) yields coimmunoadsorption of only STAT1. These data suggest that binding of either STAT2 or STAT3 to STAT1 may prevent the binding of the other.

To determine if STAT2 and STAT3 compete for binding to STAT1, each protein was translated *in vitro*, and STAT2 and STAT3 were mixed with a constant amount of STAT1. As

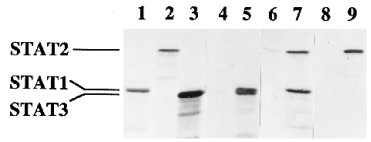

FIG. 4. **STAT1 and STAT3, but not STAT2 and STAT3, are coimmunoadsorbed after cotranslation in reticulocyte lysate.** Shown is an autoradiogram of [^{35}S]methionine-labeled proteins in aliquots of reticulocyte lysate translation mixture or immunoprecipitates prepared from the translation mixture. *Lanes 1–3*, 5 μl of STAT1, STAT2, and STAT3 translation mixtures, respectively; *lanes 4* and *5*, STAT1 and STAT3 cotranslated in the same aliquot of reticulocyte lysate, which was then immunoadsorbed with nonimmune serum or anti-STAT3 serum; *lanes 6* and *7*, STAT1 and STAT2 cotranslation immunoadsorbed with nonimmune or anti-STAT2 serum; *lanes 8* and *9*, STAT2 and STAT3 cotranslation immunoadsorbed with nonimmune or anti-STAT2 serum.

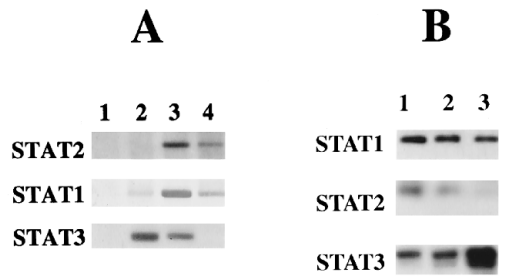

FIG. 5. **STAT1 is present in complexes with STAT2 or STAT3 but not both proteins simultaneously, and STAT2 and STAT3 compete for association with STAT1.** *A*, STAT2 and STAT3 are in separate complexes with STAT1. 3T3 cells were ruptured in hypotonic buffer and 200-μl aliquots of cytosol were immunoadsorbed with nonimmune serum (*lane 1*), anti-STAT3 (*lane 2*), anti-STAT1 (*lane 3*), or anti-STAT2 (*lane 4*), and immunoadsorbed proteins were detected by immunoblotting. *B*, STAT3 competes for STAT2 binding to STAT1. The three STATs were translated separately in reticulocyte lysate. STAT2 and STAT3 were then mixed in a ratio of 1:1 (*lane 1*), 1:2 (*lane 2*), or 1:10 (*lane 3*) prior to being mixed with a fixed amount of STAT1. The volumes of each reaction were equalized using untranslated reticulocyte extract. After 30 min on ice, the mixtures were immunoadsorbed with anti-STAT1 serum, and immunoadsorbed proteins were detected by immunoblotting.

shown in Fig. 5*B*, mixing of increasing amounts of STAT3 with STAT2 resulted in more STAT3 and less STAT2 being coimmunoadsorbed with STAT1 (*c.f. lanes 1* and *3*). As expected, the same amount of STAT1 was immunoadsorbed from each reaction.

The Y701F Mutant of STAT1 Forms a Heterocomplex with STAT2—It is known that phosphorylation of Tyr^{701} of STAT1 by JAK is necessary for interferon-induced STAT1 nuclear translocation and gene activation (23). The experiment of Fig. 6 shows that the tyrosine at position 701 of STAT1 is not necessary for STAT1-STAT2 heterodimer formation. In this experiment, the Y701F mutant of STAT1 described by Shuai *et al.* (19) was cotranslated with STAT2. As shown in the figure, immunoadsorption of the cotranslation mixture with anti-STAT1 yields coadsorption of STAT2 (*lanes 9* and *10*), indicating normal heterodimer formation (c.f. *lane 10* with *lane 8*, which contains wild-type STAT1 immunoadsorbed under the same conditions).

DISCUSSION

The notion that interferon-α/β treatment induces the formation in the cytoplasm of STAT1-STAT2 heterocomplexes is part of the developing basic model of signal transduction via STAT proteins (1, 2). The data presented in Fig. 1, however, are consistent with a variation of the model in which STAT1 and STAT2 may be preassociated as a heterodimer, with IFN-α or IFN-β treatment increasing the stability of the complex, as indicated by its resistance to dissociation by the detergent/salt

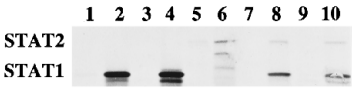

FIG. 6. **Mutation of Tyr[701] of STAT1 to phenylalanine does not affect formation of the heterocomplex with STAT2.** Wild-type STAT1, the Y701F STAT1 mutant, and STAT2 were translated singly or in combination in reticulocyte lysate with [^{35}S]methionine, and the STAT proteins were immunoadsorbed. *Lanes 1* and *2*, wild-type STAT1 translation mix immunoadsorbed with nonimmune serum or anti-STAT1; *lanes 3* and *4*, Y701F STAT1 translation immunoadsorbed with nonimmune serum or anti-STAT1; *lanes 5* and *6*, STAT2 translation immunoadsorbed with nonimmune serum or anti-STAT2, *lanes 7* and *8*, wild-type STAT1 and STAT2 cotranslation immunoadsorbed with nonimmune serum or anti-STAT1; *lanes 9* and *10*, Y701F STAT1 and STAT2 cotranslation immunoadsorbed with nonimmune or anti-STAT1.

conditions of a radioimmune precipitation assay buffer. Phosphorylation of Tyr[701] on STAT1 is required for its nuclear translocation (19), but phosphorylation does not seem to be required for STAT1 to form at least the low affinity complex with STAT2, as indicated by the observation that the Y701F mutant binds to STAT2 in reticulocyte lysate (Fig. 6). It seems likely that Tyr[701] phosphorylation is required for the high affinity, detergent/salt-resistant STAT1-STAT2 complex demonstrated in Fig. 1.

STAT1 and STAT2 appear to bind to each other when mixed in solution (Fig. 2), as do STAT1 and STAT3 (Fig. 4). Although it is reduced, the STAT1-STAT3 complex is not eliminated under the harsh conditions of the detergent/salt buffer (Fig. 3), indicating that it is of higher affinity than the STAT1-STAT2 complex shown in Fig. 1. At this time, it is unclear whether STAT protein phosphorylation as a result of LIF treatment affects the affinity of the STAT1-STAT3 interaction. At least, under our experimental conditions, we have not observed a LIF effect on the salt sensitivity of the heterocomplex. It is interesting that immunoadsorption of STAT2 or STAT3 yields coimmunoadsorption of STAT1 but not each other (Fig. 5*A*). Although this could be explained by the location of epitopes for the immunoprecipitating antibodies in a STAT2/STAT3 interaction site, it is more likely that STAT2 and STAT3 form separate heterocomplexes with STAT1. As the binding of one of these STATs to STAT1 seems to preclude binding of the other, the binding sites may overlap, or there may be a common binding site on STAT1 for the other STAT proteins as suggested by the competition experiment of Fig. 5*B*.

The work we report here raises the notion that STAT protein heterocomplexes preexist in the cytoplasm. It also has been shown by coimmunoadsorption that JAK kinases are constitutively associated with several members of the cytokine receptor superfamily (20, 21), and it is possible that the STAT protein heterodimers are associated, albeit weakly, with this receptor-attached multiprotein structure. Such a notion is consistent with the fact that STATs can be activated *in vitro* using only plasma membrane-enriched fractions of a variety of cultured cells (22) and with recent evidence of Stahl *et al.* (23) that direct interactions exist between STATs and modular tyrosine-based motifs in a number of cytokine receptors. One could then conceive of signals being passed via the phosphorylation of entirely preassociated proteins. Whether the STAT proteins then move through the cytoplasm by diffusion or in association with a general protein movement system, as suggested for the steroid receptors (24), is unknown.

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