Dominant Negative Stat3 Mutant Inhibits Interleukin-6-induced Jak-STAT Signal Transduction*

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Allard Kaptein, Valérie Paillard, and Michael Saunders
From the Laboratoires Glaxo-Wellcome, Centre de Recherche, ZA de Courtabœuf, 25 avenue du Québec, 91951 Les Ulis Cedex, France

Interleukin-6 (IL-6) induces tyrosine phosphorylation and activation of the latent transcription factor Stat3 in HepG2 cells. Mutation of Stat3 tyrosine 705 to phenylalanine (Y705F) inhibits IL-6-induced tyrosine phosphorylation of this Stat3 mutant in transfected HepG2 cells. In cotransfections of HepG2 cells, the Stat3 mutant Y705F causes a reduction of the tyrosine phosphorylation of wild type Stat3-FLAG. Moreover, Y705F inhibits the action of endogenous Stat3 in cotransfected cells, reducing IL-6 induction of a Stat3-responsive reporter construct. Y705F therefore acts as a dominant negative mutation of Stat3.

Cytokines and growth factors affect target cells through attachment to specific cell surface receptors with subsequent formation of receptor complexes and transduction of signals into the interior of the cell (1). One signal transduction pathway that leads to induction of target gene expression after cytokine stimulation is the recently described J ansus kinase (J ak)-STAT pathway (for reviews, see Refs. 2–4). Although the cytoplasmic domains of cytokine receptors lack apparent enzymatic activity, ligand binding activates associated J ak tyrosine kinases, which phosphorylate each other as well as target tyrosines in the receptor chains (5–7). Inactive cytoplasmic transcription factors or STATs are then selectively recruited to tyrosines in the receptor chains (5–7). Inactive cytoplasmic transcription factors or STATs are then selectively recruited to the receptor chains via interaction between the SH2 domain of the STATs and the receptor tyrosine phosphopeptides (2–4). Recruited STATs are phosphorylated on a specific tyrosine residue and then dissociate from the receptor, form homo- and heterodimers that translocate to the nucleus, bind to cognate DNA response elements, and activate target gene transcription (2–4).

Stat3 can be activated by a number of different cytokines and growth factors, including the IL-6 family of cytokines (IL-6, IL-11, ciliary neurotrophic factor, oncostatin M, and leukemia inhibitory factor) that share the common gp130 receptor subunit, and epidermal growth factor (1, 8, 9). Stat3 is activated through specific receptor phosphopeptide sequences with the consensus sequence Y*-X-X-Q, which is present four times in the gp130 receptor chain as well as twice in the leukemia inhibitory factor receptor chain and twice in the epidermal growth factor receptor (10–12).

We report here the construction of a dominant negative form of Stat3 capable of competitively interfering with wild type Stat3 activation by IL-6. This dominant negative mutation will prove to be a useful tool to investigate the role of Stat3 in cytokine-dependent induction of target genes.

MATERIALS AND METHODS

Stat3 Constructions—Human Stat3 cDNA was inserted in the expression vector pTL1, a derivative of pSG5 (13). The Stat3 mutation Y705F was constructed by a two-step polymerase chain reaction procedure changing the TAC codon for tyrosine, at position 705, to TTC (Flag) inserted just prior to the stop codon (encoding the amino acids DYKDDDK). Expression of the FLAG recombinant protein was verified by Western analysis with M2 anti-FLAG antibody. All constructions were verified by DNA sequencing.

Cell Culture—The HepG2 cell line was obtained from the ATCC. The cells were cultured in Eagle’s basal medium supplemented with 10% fetal calf serum (Life Technologies, Inc.), 2 mM glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml). 6 x 10⁵ cells were seeded/10 cm dish, for transfections or immunoprecipitations. IL-6 was purchased from R & D Systems.

Transcriptional Studies—HepG2 cells were transiently transfected with the calcium phosphate procedure (as described in Ref. 14). IIP6-tk-cSPAP was used for gene reporter studies. A control reporter plasmid RSV-lactamase was cotransfected in the gene reporter studies. The lactamase gene product is secreted into the medium along with secreted plasental alkaline phosphatase (SPAP). When indicated in the text Stat3, Y705F, or empty (pTL1) expression vectors were cotransfected with the reporters. SPAP and lactamase were quantitated by colorimetric assays (15). Cells were transfected in 10-cm dishes, and the next day they were trypsinized and replated on 96 wells (30,000 cells/well). After 24 or 48 h, IL-6 was added at varying doses for indicated periods of time prior to SPAP and lactamase assays. SPAP activity was normalized against lactamase activity as a control for interwell variation. Individual time points were performed in quadruplicate.

Immunoprecipitation/Immunoblotting—HepG2 cells were cotransfected with DNA (30 μg x 10⁵ cells) using the calcium phosphate procedure. After 14–16 h of incubation with the precipitate, cells were maintained on standard medium for 2 days to allow the expression of Stat3, Y705F, Stat3-FLAG, or Y705F-FLAG. Following this incubation cells were incubated overnight on RPMI 1640 containing 1% fetal bovine serum. The Hep G2 cells were then treated for 10 min with the latter medium in the presence or absence of IL-6 (25 ng/ml), washed twice with phosphate-buffered saline before being lysed for 20 min in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 15% glycerol, 1% SDS, 1% Na3VO4, 1% NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). After a pre-treatment of the cell lysate with protein G-Sepharose IgG (anti-FLAG), the lysate was incubated with the antibody M2 against the FLAG-tagged protein, which was precipitated using protein G-Sepharose. Precipitates were washed five times with lysis buffer, without Nonidet P-40 and glycerol, then boiled for 3 min in sample loading buffer (50 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 0.05% bromphenol blue, 5% b-mercaptoethanol). Precipitates were subjected to SDS-PAGE (8% gel), transferred to nitrocellulose membranes and probed with the antibody indicated in the figures. Anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology, Inc.; anti-FLAG M2 monoclonal antibody was obtained from Kodak S15.

RESULTS

Phosphorylation of Tyrosine 705 in Stat3—On the basis of alignment with Stat1, in which tyrosine 701 is the residue phosphorylated upon IFN-γ treatment (8, 16, 17), we have
identified tyrosine 705 of Stat3 as the likely site of phosphorylation by Jak kinases during signal transduction. To confirm the importance of tyrosine 705 in Stat3 activation, a mutation was made in which this tyrosine has been replaced by a phenylalanine (Fig. 1, mutation Y705F). We then determined whether Stat3 and Y705F were tyrosine-phosphorylated in HepG2 cells after IL-6 treatment. For this experiment expression plasmids were constructed containing the Stat3 or Y705F cDNA tagged at the carboxyl-terminal site with the FLAG epitope (Fig. 1). HepG2 cells were transiently transfected with expression vectors for Stat3-FLAG or Y705F-FLAG, and subsequently treated with IL-6. The M2 monoclonal antibody directed against the FLAG epitope, was used to specifically immunoprecipitate the expressed FLAG-tagged protein, but not endogenous STATs. As illustrated in Fig. 2A (M2 anti-FLAG immunoblot), Stat3-FLAG- and Y705F-FLAG-tagged proteins are expressed and immunoprecipitated. In HepG2 cells transfected with Stat3-FLAG, treatment with IL-6 resulted in a clear stimulation of the amount of tyrosine-phosphorylated protein (Fig. 2A, anti-Tyr(P) immunoblot). In untreated HepG2 cells transfected with Stat3-FLAG a very weak tyrosine phosphorylated Stat3-FLAG is observed, indicating that a low amount of phosphorylated Stat3 is constitutively present. In cells transfected with Y705F-FLAG and treated with IL-6, only a low level of tyrosine phosphorylated protein is observed. This suggests that tyrosine at position 705 of Stat3 is phosphorylated in response to IL-6 treatment. However, the faint band revealed by the anti-phosphotyrosine antibody in the IL-6-treated Y705F lane in Fig. 2A suggests that Y705F-FLAG might be weakly tyrosine-phosphorylated at an additional site following IL-6 treatment (either within the FLAG epitope, DYKDDDDK, that includes a tyrosine residue, or on an endogenous tyrosine).

**Y705F Inhibits Tyrosine Phosphorylation of Stat3-FLAG**—We next investigated whether Y705F could inhibit phosphorylation of endogenous Stat3 following IL-6 stimulation of transfected HepG2 cells. HepG2 cells were cotransfected with expression vectors containing the cDNA for Stat3-FLAG in combination with a 10-fold excess of either the empty expression vector pTL1 or Y705F (without the FLAG epitope). Using M2 anti-FLAG antibody, wild type Stat3-FLAG is precipitated (see Fig. 2B, M2 anti-FLAG immunoblot). In the cotransfection with pTL1, Stat3 is still phosphorylated upon IL-6 treatment (Fig. 2B, anti-Tyr(P) immunoblot). However, in the co-transfection with Y705F, although Stat3-FLAG is expressed normally, only a weak band is observed in IL-6 treated cells in the anti-Tyr(P) immunoblot, showing that phosphorylation of Stat3-FLAG is inhibited. This indicates that the Stat3 mutant Y705F acts in a dominant negative manner to inhibit Stat3 phosphorylation and activation. Transfection of increasing amounts of Y705F gives a dose-dependent decrease in the phosphorylation of Stat3 (data not shown), suggesting that Y705F competitively inhibits Stat3 phosphorylation by competing for the binding to the phosphotyrosine(s) on gp130.

**Y705F Inhibits Induction of an IL-6-responsive SPAP Reporter**—An IL-6-responsive reporter construct, IIP6-tk-cSPAP containing six copies of the palindromic sequence TTCCGGGAA placed upstream of the minimal thymidine kinase promoter and the SPAP cDNA sequence was used to study IL-6 signaling via Stat3. The palindromic sequence chosen has been reported as the sequence with the highest affinity in binding of phosphorylated Stat3 (18). Electrophoretic mobility shift assay studies using an oligonucleotide containing this palindromic sequence together with nuclear extracts from HepG2 cells treated or not with IL-6 reveal the presence of three complexes that correspond to Stat3 homodimers, Stat1-Stat3 heterodimers and Stat1 homodimers (data not shown, and Ref. 18).

Co-transfection of the IIP6-tk-cSPAP reporter construct and the Stat3 expression vector in HepG2 cells resulted in a more elevated promoter activation by IL-6, measured in a SPAP assay, as compared to HepG2 cells co-transfected with the empty expression vector pTL1 and the SPAP reporter construct (Fig. 3A, IL-6 dose response, 9.4-fold versus 5.6-fold maximal induction; and 3B, time course after IL-6 addition). When Y705F was transfected in HepG2 cells a strong inhibition is observed in the IL-6-induced promoter activation (Fig. 3A, activation is reduced to ~2-fold). These results indicate that phosphorylation of tyrosine at position 705 of Stat3 is essential in IL-6 signaling and that the mutant Y705F reduces the signal transduction via endogenous Stat3. Transfection of HepG2 cells with increasing amounts of Y705F expression vector inhibits IL-6 induction of the SPAP reporter in a dose-dependent manner, indicating competition of Y705F with endogenous Stat3 upon IL-6 activation (Fig. 4). Stat3 and Y705F have no effect on expression of a cotransfected control reporter construct RSV-lactamase whose expression remains unaltered by IL-6 treatment (data not shown).

**DISCUSSION**

Mutation of tyrosine 705 to phenylalanine in Stat3 leads to reduced levels of tyrosine-phosphorylated mutant protein following IL-6 treatment, indicating that this residue is a site of phosphorylation. This is in accordance with the alignment with other members of the Stat family, in particular Stat1, where tyrosine 701, and Stat2 where tyrosine 690 are phosphorylated, respectively, following cytokine treatment (16, 17, 27). More-
over, immunoprecipitation studies show that Y705F inhibits phosphorylation of Stat3-FLAG following IL-6 treatment in cotransfected HepG2 cells (Fig. 2B). In addition, the Y705F mutation of Stat3 acts in a dominant negative manner to inhibit IL-6 activation of the IL6-tk-cSPAP reporter construct in HepG2 cells (Fig. 3). Y705F competitively inhibits IL-6 signal transduction as measured in our transcriptional assay (Fig. 4) and in immunoprecipitation experiments measuring Stat3-FLAG tyrosine phosphorylation in cotransfections with increasing quantities of Y705F (data not shown). This demonstrates that tyrosine 705 in Stat3 is critical for activation by IL-6.

Recent studies indicate that Stat1 and Stat6 bind directly to tyrosine phosphopeptides derived from the IFN-γ receptor and the IL-4 receptor, respectively (19, 20). Current models suggest that inactive cytoplasmic STATs are recruited to the activated receptor by docking of the STAT SH2 domain to selected receptor tyrosine phosphopeptides, where they are in turn phosphorylated on a single tyrosine by Jak kinases (10, 19, 20). Stat1 is phosphorylated on Tyr-701, and mutation of this tyrosine to phenylalanine inactivates this mutant Stat1 (16). Domain swapping experiments between Stat1 and Stat2 demonstrate that the SH2 domain is critical for selective recruitment to activated receptor chains (21).

The amino acid sequences surrounding the phosphorylated tyrosine dictate substrate recruitment via specificity of SH2 domain/tyrosine phosphopeptide interactions (Ref. 22; see Ref. 23 for review). Recent work indicates that Stat3 can be activated via receptor tyrosine phosphopeptides with glutamine in the +3 position (Y*XXQ) (10). The glutamine at +3 is believed to play a critical role in Stat3 recruitment since its alteration to alanine prevents Stat3 tyrosine phosphorylation in chimeric receptor constructs (10). Interestingly, this target sequence differs from the internal Stat3 tyrosine phosphopeptide sequence that may be used in back-to-back Stat3 homodimer formation (Y*LKT). This finding also applies to Stat1α, which is recruited by a Y*DKPH motif on the IFN-γ receptor (19, 24), but when forming a homodimer binds to Y*IKTE (E) and in heterodimers with Stat3 may bind Y*LKT(K). The tyrosine 705 residue that is phosphorylated during Stat3 activation is thought to be immediately distal to the carboxy boundary of the SH2 domain, a distance too short to allow an intramolecular interaction between the SH2 domain and the phosphorytrosine residue (25). This close proximity suggests that phosphorylation of tyrosine 705 may lead to a conformational change of the Stat3 SH2 domain that alters its selectivity for tyrosine phosphopeptides. After tyrosine phosphorylation, such an induced conformational change might encourage dissociation of Stat3 from the receptor and enhance formation of Stat dimers by altering selectivity from Y*XXQ, the receptor sites that are likely to be bound by non-activated Stat3, to Y*LKT the tyrosine phosphopeptide present in Stat3 that allows STAT dimerization by interacting with the SH2 domain of the dimeric partner. Such an alteration in the selectivity of an SH2 domain for target tyrosine phosphopeptides has been reported for the tyrosine kinase p56lck (26).

Our current model for the dominant negative action of Y705F (Fig. 5) implies that the mutated Stat3 can still bind to IL-6-activated gp130 tyrosine phosphopeptides, since the SH2 domain is unaltered, but the critical residue at position 705 can no longer be tyrosine-phosphorylated by Jak kinases. Consequently, Y705F may block recruitment of Stat3 to receptor phosphopeptide docking sites and hence prevent activation of wild type Stat3. If phosphorylation of tyrosine 705 of Stat3 leads to a conformational change of its SH2 domain leading to reduced affinity for the receptor docking sites, then the Stat3 mutant Y705F/receptor complex may have a longer half-life than its wild type homolog.

It cannot be excluded that Y705F may also form heterodimers with activated Stat3 through a single SH2/tyrosine interaction (Fig. 5B, bottom). Such putative heterodimers with weakened molecular interactions may be susceptible to phosphatase deactivation or may simply be unable to bind DNA target sequences.

By altering the tyrosine residue that is phosphorylated in the different STATs, it should be possible to create similar dominant negative mutations, that will allow investigation of the
role of individual STATs in the cellular responses to various cytokines and growth factors.

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FIG. 5. Model for the mode of action of the dominant negative Stat3 mutation Y705F. A, activation of wild type Stat3. IL-6 treatment causes Stat3 recruitment to receptor tyrosine phosphopeptides (gp130) where it is phosphorylated on tyrosine 705 (Y) by jak kinase. Stat3 dissociates from the receptor, forms dimers, and migrates to the nucleus where it binds response elements in target genes. Stat3 is shown schematically. B, two possible levels of dominant negative activity of Y705F. Y705F may compete with Stat3 for binding to the tyrosine phosphopeptides on gp130, and when in excess exclude Stat3 recruitment. Y705F may also be able to form a non-functional weak heterodimer with activated Stat3.
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