Targeted Inhibition of Interferon-γ-dependent Intercellular Adhesion Molecule-1 (ICAM-1) Expression Using Dominant-Negative Stat1*

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A subset of epithelial immune-response genes (including intercellular adhesion molecule-1 (ICAM-1)) depends on an IFN-γ signal transduction pathway with the Stat1 transcription factor as a critical intermediate. Excessive local activation of this pathway may lead to airway inflammation, so we sought to selectively down-regulate the pathway using a dominant-negative strategy for inhibition of epithelial Stat1 in a primary culture airway epithelial cell model. Using a Stat1-deficient cell line, we demonstrated that transfection of wild-type Stat1 expression plasmid restored appropriate Stat1 expression and IFN-γ-dependent phosphorylation as well as consequent IFN-γ activation of cotransfected ICAM-1 promoter constructs and endogenous ICAM-1 gene expression. However, mutations of Stat1 at Tyr-701 (JAK kinase phosphorylation site), Glu-428/429 (putative DNA-binding site), His-713 (splice site resulting in Stat1β formation), or Ser-727 (MAP kinase phosphorylation site) all decreased Stat1 capacity to activate the ICAM-1 promoter. The Tyr-701 mutant (followed by the His-713 mutant) were most effective in disabling Stat1 function and in overcoming the activating effect of cotransfected wild-type Stat1 in this cell system thereby highlighting the effectiveness of blocking Stat1 homo- and hetero-dimerization. In experiments using primary culture human tracheobronchial epithelial cells (hTBECs) and each of the four Stat1 mutant plasmids, transfection with the Tyr-701 and His-713 mutants again most effectively inhibited IFN-γ activation of an ICAM-1 gene promoter construct. Then by transfecting hTBECs with wild-type or mutant Stat1 tagged with a Flag reporter sequence, we used dual immunofluorescence to show that hTBECs expressing the Tyr-701 or His-713 mutants were prevented from expressing endogenous ICAM-1 in response to IFN-γ treatment. The capacity of a specific Stat1 mutations to exert a potent dominant-negative effect on IFN-γ signal transduction provides for further definition of Stat1 structure function and a means for natural or engineered expression of mutant Stat1 to selectively down-regulate activity of this pathway in a cell type- or tissue-specific manner during immune and/or inflammatory responses.

Transcription factors generally contain at least two independent domains for DNA binding and for activation of transcription (1). Removal of the transactivation domain has been shown in many cases to result in an inactive factor that can bind and displace wild-type protein thereby creating a dominant-negative action (2). Targeting such a dominant-negative construct so that it is expressed in a specific tissue has been useful in understanding the function of specific transcription factors in different tissues. Accordingly, this strategy offers an advantage over complete deletion of the factor by homologous recombination with the endogenous gene if there is a goal of defining function in a specific tissue or cell type. In that context, we have determined that epithelial barrier tissue (and airway epithelial cells in particular) selectively activate a subset of immune response genes to mediate immunity and inflammation, and this activation is controlled by the Stat1 transcription factor (3, 4). Thus, the present experiments were aimed at establishing a dominant-negative strategy for investigating the action of epithelial Stat1.

In defining this strategy, it is noteworthy that STAT1 family proteins are somewhat more complex than other transcription factors. The STAT proteins act as critical intermediates in cytokine-dependent gene activation based on their dual capacities for signal transduction (at the cell surface) and activation of transcription (in the nucleus) (5). Signal transduction depends on programmed assembly of cytokine receptors, receptor-associated JAK kinases, and in some cases serine kinases, that recruit and activate specific STAT proteins (6–8). Phosphorylated/activated STATs then dimerize, translocate to the nucleus, and direct transcription of specific target genes. In particular, the first member of the STAT family (designated Stat1) is critical for IFN-dependent gene activation (9). IFN-γ-dependent oligomerization of the IFN-γ receptor and consequent cross-phosphorylation of receptor-associated Jak1 and Jak2 kinases and the receptor α-chain leads to SH2-dependent recruitment of Stat1 (6, 10). Stat1 then undergoes Tyr-701 phosphorylation and SH3-dependent release from the receptor as a homodimer (10, 11) that can translocate to the nucleus and bind to a specific inverted repeat DNA element (3, 12, 13). Amplification steps in this pathway responsible for further specificity include MAP kinase-dependent phosphorylation of Ser-727 in the cytoplasm.

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‡ The abbreviations used are: STAT, signal transducer and activator of transcription; hTBEC, human tracheobronchial epithelial cell; ICAM-1, intercellular adhesion molecule-1; IRE, IFN-γ response element; JAK, Janus family tyrosine kinase; Ab, antibody; mAb, monoclonal Ab; MAP, mitogen-activated protein; PCR, polymerase chain reaction; RANTES, regulated upon activation, normal T cell expressed and secreted; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; bp, base pair(s); MES, 4-morpholineethanesulfonic acid.
Dominant-Negative Stat1

IFN-α assessment of tissue-specific alterations in Stat1 activity. In ever, as noted above, this approach does not allow for functional biologic responses, especially viral immunity (15, 16). However, recent studies of Stat1 biology in the genetically Stat1−/− relationships and a primary culture human airway epithelial cell should prove useful for regulating IFN-α-driven Stat1 in a biologically relevant human cell model. The mutants knowledge, this represents the initial indication that loss of nonfunctional (in a Stat1-deficient cell line) and may exert a Stat1 transactivation domain (the carboxyl-terminal 38 amino acids (17–19). Thus, the level of Stat1-dependent gene activation may critically control immune and inflammatory responses, and regulating the level of epithelial Stat1 activity may influence the type of host response.

Accordingly, we sought a means to down-regulate the IFN-α-driven signal transduction pathway using a dominant-negative strategy for Stat1 in a relevant ex vivo system. We took advantage of Stat1 (and other STAT protein) structure-function relationships and a primary culture human airway epithelial cell model with a defined IFN-γ-dependent immune response gene (ICAM-1) to establish strategies for inhibition of Stat1 activity. We reasoned that mutations that may inactivate a potential Stat1 DNA-binding site at Glu-428 and Glu-429 (20), the Jak1/2 phosphorylation site at Tyr-701 (21), or the MAP kinase phosphorylation site at Ser-727 (14) or deletion of the putative Stat1 transactivation domain (the carboxyl-terminal 38 amino acids) (22) might each serve to generate a Stat1 protein that is nonfunctional (in a Stat1-deficient cell line) and may exert a dominant-negative action (in an epithelial cell model). To our knowledge, this represents the initial indication that loss of function may correlate with dominant-negative activity for Stat1 in a biologically relevant human cell model. The mutants should prove useful for regulating IFN-γ-driven gene expression in a tissue- or cell-specific manner and for further analyzing structure-function in the Stat1 pathway.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human IFN-γ was from Genentech (San Francisco, CA); rabbit antisera to Stat1 (p91) and to Stat1 and β (p91) were from J. E. Darnell, Jr. (Rockefeller University, New York); mouse anti-Stat1 IgG1 Ab conjugated to agarose was from Santa Cruz Biotechnology (Santa Cruz, CA); goat anti-mouse IgG, Ab conjugated to horseradish peroxidase was from Boehringer Mannheim; mouse anti-Stat1 (ISGF3) IgG2a, and anti-Stat3 IgG1, mAb and anti-phosphothreonine IgG1, Ab conjugated to horseradish peroxidase was from Transduction Laboratories (Lexington, KY); mouse anti-Flag M2 IgG1, Ab unconjugated or conjugated to biotin was from Eastman Kodak Co.; mouse anti-ICAM-1 mAb LB2 was from E. Clark (University of Washington); mouse anti-ICAM-1 mAb 84H10 was from Immunotech (Westbrook, ME); and donkey anti-rabbit Ab conjugated to indocarbocyanine (Cy3) or fluorescein (FITC), donkey anti-mouse Ab conjugated to tetramethyl-rhodamine (TRITC), goat anti-mouse IgG (H + L) Flab/’2 conjugated to Cy3, and FITC-conjugated streptavidin were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Cell Culture—Stat1-deficient U93 cells were obtained from G. Stark (Cleveland Clinic, OH) and I. Kerr (Imperial Cancer Research Foundation, London) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum, 1-glutamine and penicillin/streptomycin as described previously (23). Human tracheobronchial epithelial cells (hTBEcs) were isolated from mucosal strips by enzymatic dissociation and then cultured in LHC-8 medium on flasks coated with collagen/albumin as described previously (3, 4, 24).

Wild-type Stat1 Expression Plasmids—The human Stat1 cDNA (en- coding the 750 amino acid Stat1 protein) in pBluescript II SK (pSK-Stat1) was a gift from J. E. Darnell, Jr. Our expression vector for Stat1 was generated in four steps: (i) the Stat1 5′-UTR was removed from pSK-Stat1 by SacII/NcoI digestion, releasing the 5′-UTR plus sequence encoding Stat1 amino acids 1–27. A double-stranded oligonucleotide containing SacII and EcoRI sites at the upstream Stat1 sequence encoding amino acids 1–27 including the adjacent downstream NcoI site was then inserted into SacII/NcoI-digested pSK-Stat1 generating pSK-Stat1−5UTR; (ii) the Stat1 3′-UTR was removed by modifying the Lex A containing expression plasmid pBXL3 (a gift from P. Broad, Zeneca Pharmaceutical, Cheshire, United Kingdom). A double-stranded oligonucleotide containing a BamHI site at the 3′-UTR downstream end and Stat1 sequence encoding amino acids 743–750 including the adjacent upstream EcoRI site was inserted into EcoRI/BamHI-digested pBXL3 generating pBXL3-Stat1(743–750); (iii) pSK-Stat1−5UTR was digested with EcoRI releasing Stat1 sequence encoding amino acids 1–742, and this sequence was inserted into EcoRI-digested pBXL3-Stat1(743–750) generating pBXL3-Stat1; and (iv) the Lex A cDNA was removed by SalI/NcoI-digestion of pBXL3-Stat1 and insertion of a double-stranded oligonucleotide containing SacII, HindIII, and NcoI sites, the Lex A 5′-UTR, ApaI and SacII sites, and Stat1 sequence encoding amino acids 1–27 including the adjacent downstream NcoI site generating pBXS-Stat1 (in which the Stat1 coding sequence is driven by the SV40 early region).

To insert the Stat1 coding sequence downstream to the more potent CMV promoter, pcDNA3 (Invitrogen, San Diego, CA) was modified by digestion with HindIII/Xbal and insertion of a double-stranded oligonucleotide containing an upstream HindIII overhang that destroyed the HindIII site, KpnI, SacII, EcoRV, BamHI, NotI, and BspEI sites, and a downstream Xbal overhang that destroyed the Xbal site generating pcDNA3m. The Stat1 sequence encoding amino acids 1–750 from pBX- Stat1 was released by SacII/BamHI digestion and this sequence was inserted into pBII/BamHI-digested pcDNA3m generating pcDNA3m-Stat1. To add the 8 amino acid Flag sequence (25) downstream to the Stat1-coding sequence, a double-stranded oligonucleotide containing downstream BamHI and EcoRV sites, a stop codon, and sequence encoding the Flag peptide and Stat1 amino acids 743–750 including the adjacent upstream EcoRI site was inserted into BamHI/EcoRI-digested pcDNA3m-Stat1 generating pcDNA3m-FlagStat1. To add the double-stranded oligonucleotide containing downstream BamHI and EcoRV sites, a stop codon, and sequence encoding the Flag peptide and Stat1 amino acids 743–750 including the adjacent upstream EcoRI site was inserted into BamHI/EcoRI-digested pcDNA3m-Stat1 generating pcDNA3m-FlagStat1.

Mutant Stat1/Expression Plasmids—Selected mutations in the Stat1 coding sequence were created using the Stat1 cDNA as template in the polymerase chain reaction (PCR) with Thermo flavus DNA polymerase (Amersham Corp.) and a 55°C annealing cycle. All PCR generated fragments were sequenced using the dyeoxynucleotide technique to verify sequence integrity (26).

To mutate Stat1 at amino acids 428 and 429 (the putative DNA-binding site), an upstream primer encoding Stat1 sequence extending from the SpalI site at the codon for amino acid 323 to 20 bp downstream and a downstream primer extending from the Espl site at the codon for amino acid 446 to 74 bp upstream (with the codons for amino acids 428 and 429 converted from glutamine to alanine) were used. The PCR product was inserted into pSpuI/EcoRI-digested pBXL3-Stat1−5UTR, generating pSpuI/ EcolRI-Glu428/9M. To insert this mutant Stat1 sequence downstream to the CMV promoter, pSK-Stat1−5UTR-Glu428/9M was digested with PstI/SacII and the resulting Stat1 sequence encoding amino acids 1 to 472 with the mutations of amino acids 428 and 429 was inserted into PstI/SacII-digested pcDNA3m-Stat1 generating pcDNA3m-FlagStat1.

To mutate Stat1 at amino acids 428 and 429 (the putative DNA-binding site), an upstream primer encoding Stat1 sequence extending from the SpalI site at the codon for amino acid 323 to 20 bp downstream and a downstream primer extending from the Espl site at the codon for amino acid 446 to 74 bp upstream (with the codons for amino acids 428 and 429 converted from glutamine to alanine) were used. The PCR product was inserted into pSpuI/EcoRI-digested pBXL3-Stat1−5UTR, generating pSpuI/ EcolRI-Glu428/9M. To insert this mutant Stat1 sequence downstream to the CMV promoter, pSK-Stat1−5UTR-Glu428/9M was digested with PstI/SacII and the resulting Stat1 sequence encoding amino acids 1 to 472 with the mutations of amino acids 428 and 429 was inserted into PstI/SacII-digested pcDNA3m-Stat1 generating pcDNA3m-FlagStat1.

To mutate Stat1 at amino acids 701 (the Jak1/2 phosphorylation site), an upstream primer encoding Stat1 sequence extending from the SpalI site at the codon for amino acid 701 converted from tyrosine to phenylalanine (27) were used. The PCR product was inserted into pSpuI/EcoRI-digested pBXL3-Stat1−5UTR, generating pSpuI/ EcolRI-Glu701/702M. To insert this mutant Stat1 sequence downstream to the CMV promoter, pSK-Stat1−5UTR-Glu701/702M was digested with PstI/MluI and the resulting Stat1 sequence encoding amino acids 1 to 472 with the mutations of amino acids 701 was inserted into PstI/MluI-digested pcDNA3m-Stat1 generating pcDNA3m-FlagStat1.
The Flag sequence was inserted downstream to the mutant Stat1 sequence in pcDNA3m-Stat1-Tyr701M to generate pcDNA3m-Stat1-Tyr701M/Flag.

To delete Stat1 sequence encoding amino acids 712 to 750 (generating Stat1-deficient cell line derived by somatic mutation from HT1080 fibrosarcoma cells (23). Immunoblot analysis of newly expressed wild-type and mutant Stat1 proteins in this system using anti-Stat1 and anti-phosphoryrosine mAbs indicated high level expression of wild-type Stat1 that was appropriately phosphorylated in response to IFN-γ treatment (Fig. 1). Mutations at Thr-701 (a putative kinase phosphorylation site) gave a similar level of expression and phosphorylation as wild-type, whereas mutation at Tyr-701 correctly prevented phosphorylation. Consistent with the absence of tyrosine phosphorylation, we also demonstrated that wild-type but not Tyr-701 mutant Stat1 translocated to the nucleus in response to IFN-γ in this cell system (data not shown). Mutation at His-713 (causing truncation at Val-712) resulted in an appropriately smaller (84-kDa) Stat1β species, which was unseparated from the phosphorylated species by this electrophoresis technique (as is also true of endogenous Stat1β) (22). Each mutant Stat1 species appeared to be expressed at a similar level to the wild-type Stat1 in this expression system (consistent with similar levels of translation efficiency for each plasmid construct).

Modification of ICAM-1 Promoter Activity in Stat1-complemented Cells—To next determine the capacity of wild-type and mutant Stat1 expression plasmids to regulate IFN-γ-responsive gene activation in this same cell system, we cotransfected each of the Stat1 expression constructs with a reporter plasmid that contained the ICAM-1 gene interferon-γ-response element (IRE) driving a luciferase reporter gene (3, 4). The ICAM-1 promoter was chosen in preparation for similar experiments with hTBEC cells (as noted below).

Expression plasmids encoding wild-type Stat1 conferred IFN-γ responsiveness in this system, whereas each of the four mutations disabled Stat1 function to varying degrees (Fig. 2A). The Tyr-701 mutation was the most effective and the Ser-727 mutation was the least effective in inactivating Stat1 function (Fig. 2A). In their possible role as dominant-negative mutations, each of the four Stat1 mutants exhibited the same rank order of potency for antagonizing wild-type Stat1 function as assessed by inhibition of IRE-driven gene activation in Stat1-complemented cells with Tyr-701 and His-713 mutants as the most effective (Fig. 2B). This system was designed with a ratio of mutant to wild-type Stat1 expression plasmid of 10:1 after titration of this ratio indicated a maximal effect for the Tyr-701 and His-713 mutants (Fig. 3).
Taken together, these findings provided relatively good correlation between the failure to mediate Stat1-dependent gene activation and the capacity to antagonize wild-type Stat1-dependent gene activation. The findings also offered initial evidence that Glu-428/429 was a critical site for Stat1 action (analogous to Glu-434/435 for Stat3) (20) and that Ser-727 was less than observed for other gene promoters (14), suggesting some gene-specific differences in the importance of this site for Stat1 function.

To further determine whether Stat1 expression constructs modify endogenous ICAM-1 promoter activity in U3A cells, we also monitored ICAM-1 expression that is transcriptionally induced by IFN-γ treatment (3). Using dual immunofluorescence labeling for Stat1 and ICAM-1, we found that cells containing wild-type Stat1 (but not Stat1-Tyr-701M) expressed ICAM-1 after IFN-γ stimulation (Fig. 4). The lower level of ICAM-1 expression relative to ICAM-1 promoter activity in U3A cells (compared with hTBECs) may reflect an additional post-transcriptional influence on ICAM-1 levels in hTBECs. Each of these approaches (monitoring ICAM-1 promoter activity and tracking ICAM-1 expression) was then extended to studies of primary culture hTBECs to determine whether Stat1 mutants exerted a dominant-negative action on endogenous Stat1 in a biologically relevant cell system.

Capacity of Stat1 Mutants to Inhibit ICAM-1 Gene Promoter Activity in hTBECs—In contrast to experiments using complemented U3A cells, we now aimed to compete native Stat1 activity by overexpressing mutant Stat1 proteins using an airway epithelial cell model system (hTBECs) with well defined IFN-γ-dependent transcriptional control for the ICAM-1 gene promoter (3, 4, 24). The initial approach was similar to that used for U3A cells, in which ICAM-1 promoter activity was monitored using a cotransfected ICAM-1 luciferase reporter plasmid. As was the case for Stat1-complemented U3A cells, the Tyr-701M construct most effectively inhibited IFN-γ-dependent promoter activity (Fig. 5). The His-713M construct was nearly as effective in blocking endogenous Stat1 activity, whereas the Glu-428/429 and Ser-727M constructs had no significant effect in antagonizing Stat1 activity on ICAM-1 promoter activity. This system was designed with a ratio of expression to reporter plasmid of 1:1, after experiments with higher ratios of expression to reporter plasmid offered no ad-

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In addition, hTBECs transfected with wild-type Stat1/Flag revealed protein expression and pattern of cellular immunofluorescence. For each mutant, the expression plasmid was added in increasing amounts (0.05–0.50 μg) to give ratios of mutant to wild-type Stat1 of 1:1, 2:1, 5:1, or 10:1. After transfection, cells were incubated without (●) or with (■) IFN-γ (100 units/ml for 18 h at 37 °C) and then assayed for luciferase activity. Each value is the average for duplicate samples and is representative of three experiments.

Capacity of Stat1 Mutants to Inhibit Endogenous ICAM-1 Gene Expression in hTBECs—We next aimed to determine whether inhibition of Stat1 activity by mutant Stat1 constructs was able to also block expression of the endogenous ICAM-1 gene. We again used a strategy similar to the one used for U3A cells, but in this case, it was necessary to separate transiently expressed from native Stat1 protein. Accordingly, we generated constructs encoding for wild-type and Tyr-701M Stat1 fused to an 8-amino acid epitope (Flag) that could be used to track expression. Validation experiments in U3A cells indicated that the wild-type Stat1/Flag fusion protein was appropriately expressed, phosphorylated, translocated, and was capable of mediating activation of the ICAM-1 promoter, whereas the Stat1-Tyr-701M/Flag fusion protein was expressed at the same level but failed to undergo phosphorylation or translocation or to activate the ICAM-1 promoter and was effective in blocking wild-type Stat1 activity when transfected into cells at a ratio of mutant to wild-type Stat1 of 10:1 (Fig. 1 and data not shown).

In the case of hTBECs, expression of wild-type Stat1/Flag was again similar to wild-type Stat1 as assessed by the level of protein expression and pattern of cellular immunofluorescence. In addition, hTBECs transfected with wild-type Stat1/Flag responded to IFN-γ with a loss of the nuclear halo (indicating nuclear translocation of Stat1/Flag) and expression of ICAM-1 (indicating ICAM-1 gene activation by Stat1/Flag) (Fig. 6). By contrast, hTBECs expressing Stat1-Tyr-701M/Flag showed no change in Stat1 immunofluorescence pattern and failed to express ICAM-1 in response to IFN-γ treatment. Additional experiments with each of the other three Stat1 mutants indicated that their effects on ICAM-1 promoter activity also correlated well with effects on endogenous ICAM-1 expression (Fig. 7). Thus, the Tyr-701 and His-713 mutants but not Glu-428/9 and Ser-727 mutants were effective in blocking both ICAM-1 promoter/reporter activity and ICAM-1 expression induced by IFN-γ.

**DISCUSSION**

This report represents the initial examination of dominant-negative action for mutant Stat1 on endogenous gene expression in a primary culture cell model. In that context, however, several STAT family members have been examined for dominant-negative activities on reporter gene and in some cases endogenous gene expression in transformed cell models. Stat3 bears the closest homology to Stat1 and mutations at its DNA-binding site (Glu-434/435) or JAK kinase phosphorylation site (Tyr-705) as well as deletion of the carboxyl-terminal portion that includes the transactivation domain (yielding Stat3β) appear to suppress the activity of Stat3-responsive reporter constructs transfected into transformed cell lines (29–31). This approach required cotransfection of wild-type Stat5 to reconstitute the complete activation pathway (in COS cells) or the presence of multiple copies of the Stat3 DNA response element joined to a thymidine kinase or junB minimal promoter in the 5′ flanking region of the reporter (in HepG2 or M1 cells). Other recent reports indicate that deletion of the carboxyl-terminal transactivation domain of Stat5 also results in sustained DNA.
binding and a dominant-negative effect on reporter gene activity of Stat5-responsive constructs (in COS and Ba/F3 cell lines) and expression level of interleukin-3 early response genes (in interleukin-3-dependent Ba/F3 or 32Dc1-Epo1 wild-type cells) (32–34). In addition, an analysis of Stat2 indicates that mutations at Glu-428/9 (35). We also found that loss of function correlated closely with dominant-negative action for Stat1 in a Stat1-complemented U937 cell line. However, these correlations were less apparent in studies of endogenous gene activation and expression in primary culture human airway epithelial cells. Thus, each of these studies highlights the need for investigating STAT function in primary culture human cell models with defined pathways for STAT-dependent regulation of endogenous cytokine-dependent genes.

Our data indicate that four distinct mutations of Stat1 cause loss of function, and at least two of these mutations (Tyr-701 to Phe-701 and His-713 to a stop codon) exert a consistent dominant-negative action on reconstituted as well as endogenous IFN-γ-dependent signal transduction and consequent ICAM-1 gene expression. Stat1-Tyr-701M and Stat1-His-713M (Stat1β) are nonfunctional (as assessed by its capacities for phosphorylation, nuclear translocation, activation of the ICAM-1 promoter, or induction of ICAM-1 expression) in a Stat1-deficient cell line. These mutations also inhibit the function of endogenous wild-type Stat1 in hTBEcs and block IFN-γ-driven expression of endogenous ICAM-1. The effectiveness of the Stat1-Tyr-701 mutation is likely based on its capacity to compete with endogenous wild-type Stat1 at the level of recruitment to binding sites for heterodimeric IFN-γ/Stat1 complexes.
the activated IFN-γ receptor. Thus, Stat1 and Stat1-Tyr-701M exhibit equivalent capacities for recruitment to the phosphorylated receptor (via native SH2 domains). However, only wild-type Stat1 is then likely to be capable of dissociating from the receptor, because this dissociation step depends on Tyr-701 phosphorylation of Stat1 and consequent affinity-driven association with a companion Stat1 in preference to the IFN-γ receptor α-chain (10). The marked effectiveness of Tyr-701 mutation in down-regulating Stat1-dependent gene activation indicates that interfering with this relatively early step in IFN-γ signal transduction (i.e. Stat1 homodimerization) is critical for efficiently blocking this pathway.

In contrast to the Tyr-701 mutant, Stat1β effectively undergoes tyrosine phosphorylation with consequent homodimerization (with itself) or heterodimerization (with full-length Stat1) and translocation to the nucleus with binding to the IRE. However, once bound, the truncated version of Stat1 is incapable of activating transcription. The dominant-negative action of Stat1β appears to be based on its failure to activate transcription as a homodimer and its capacity to "decoy" full-length Stat1 (Stat1α) to form a less effective Stat1α/Stat1β heterodimer. Accordingly, the degree of blockade may depend on the relative affinities of Stat1β for Stat1 in comparison to Stat1 for itself. In fact, the slight decrease in dominant-negative activity for Stat1β (compared with the Tyr-701 mutant; Fig. 3) may reflect the possibility that phosphorylated Stat1β has a slightly higher affinity for activated Stat1 than for activated Stat1β. It is also possible that Stat1β bound to the IRE may recruit additional Stat1 through its amino-terminal end as described for promoters containing multiple IRE sites (36). In either case, Stat1β exerts significant dominant-negative activity only when its expression exceeds that for native Stat1, and maximal inhibition of Stat1 action was not observed until the ratio of Stat1β to Stat1 was 10:1. This finding argues against the possibility that Stat1β might function as a naturally occurring negative regulator of Stat1 activity until its expression levels exceed that for Stat1. To date, cellular levels of Stat1β are two- to three-fold lower than for Stat1 in airway epithelial cells and other cell types (22). However, the ratio of full-length Stat1 to Stat1β and possible dysregulation of this ratio has not yet been examined during in vivo conditions with altered activity of IFN-γ.

Mutations of the putative DNA-binding site (Glu-428 and -429) or the MAP kinase phosphorylation site (Ser-727) were examined during inflammation. Studies of Stat1-deficient mice indicate that Stat1 is required for immunity to nonrespiratory viruses (15, 16), but the role of Stat1-dependent gene activation in response to inhaled agents (infectious or allergic) or in the immune function of the airway epithelium is still uncertain. The present results imply that naturally occurring or genetically engineered defects in phosphorylation-dependent activation of Stat1 will provide the most potent insight into the role of Stat1 in mediating airway epithelial immunity and inflammation.

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