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STAT3 activation by cytokines utilizing gp130 and related transducers involves a secondary modification requiring an H7-sensitive kinase

(ciliary neurotrophic factor/interleukin 6/leukemia-inhibitory factor/oncostatin M/granulocyte colony-stimulating factor)

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ABSTRACT **Ciliary neurotrophic factor, oncostatin M, leukemia-inhibitory factor, and interleukin 6 are related cytokines that initiate signaling by homodimerizing the signal-transducing receptor component gp130 or by heterodimerizing gp130 with a gp130-related receptor component. Receptor dimerization in turn activates receptor-associated kinases of the Jak/Tyk family, resulting in the rapid tyrosine phosphorylation of several intracellular proteins, including those of two members of the signal transducers and activators of transcription (STAT) family—STAT1 and STAT3. Here we show that all cytokines that utilize gp130 sequentially induce two distinct forms of STAT3 in all responding cells examined, with the two forms apparently differing because of a time-dependent secondary serine/threonine phosphorylation involving an H7-sensitive kinase. While both STAT3 forms bind DNA and translocate to the nucleus, the striking time-dependent progression from one form to the other implies other important functional differences between the two forms. Granulocyte colony-stimulating factor, which utilizes a receptor highly related to gp130, also induces these two forms of STAT3. In contrast to a number of other cytokines and growth factors, all cytokines using gp130 and related signal transducers consistently and preferentially induce the two forms of STAT3 as compared with STAT1; this characteristic STAT activation pattern is seen regardless of which Jak/Tyk kinases are used in a particular response, consistent with the notion that the receptor components themselves are the primary determinants of which STATs are activated.**

Ciliary neurotrophic factor (CNTF), oncostatin M (OSM), leukemia-inhibitory factor (LIF), and interleukin 6 (IL-6) are related cytokines that share signal-transducing receptor components such as gp130 and a gp130-related protein initially identified as the LIF receptor (LIFR) (1–8). IL-6 initiates signaling via homodimerization of gp130, while CNTF, LIF, and OSM initiate signaling by heterodimerizing gp130 along with LIFR (5, 9, 10). OSM may in some cells utilize an alternative signal-transducing receptor component instead of LIFR (11). Constitutively associated with the intracellular domains of the signal-transducing receptor components (12, 13) are members of a family of cytoplasmic tyrosine kinases known as the Jak/Tyk kinases (14). Dimerization of the signal-transducing receptor components activates the associated Jak/Tyk kinases, resulting in the rapid tyrosine phosphorylation of these kinases, of the receptor components, and of a number of other intracellular proteins (5, 12, 15). In contrast to other cytokines that only activate particular combinations of Jak/Tyks (14, 16–20), the CNTF family of cytokines can activate all members of the Jak/Tyk family, although the selection of kinases can vary depending upon the cell line examined (12). Despite the use of different combinations of Jak/Tyks in different cell lines, all members of the CNTF family of cytokines stimulate tyrosine phosphorylation of a very similar set of proteins in all cells examined (15), consistent with the notion that the signal-transducing receptor components contain motifs that specify downstream signaling molecules to be activated by the Jak/Tyk kinases (21, 22). This selection of substrates is also largely independent of whether signaling is initiated via gp130 homodimerization or gp130/LIFR heterodimerization (15), suggesting that these two receptors have similar substrate-specifying motifs (21).

Many of the proteins inducibly tyrosine-phosphorylated by the CNTF family of cytokines correspond to previously identified signal-transducing molecules (15). One of these is STAT1 (13, 15, 23), the DNA-binding transcriptional activator first identified for its role in interferon signaling (24, 25). Recent work has revealed that IL-6, CNTF, and their relatives can activate a second molecularly cloned STAT protein (26, 27), termed STAT3, which binds DNA response elements similar to those recognized by STAT1 and apparently corresponds to a DNA-binding activity previously referred to as acute-phase response factor (APRF) (28–30). The CNTF family also induces tyrosine phosphorylation of two closely migrating proteins designated p88 and p90 that, while similar in size to STAT1 and weakly immunoreactive with antibodies to STAT1, were shown not to correspond to STAT1 (13, 15, 23); we considered the possibility that at least one of these might correspond to STAT3. Here we show that the p88 and p90 proteins correspond to different forms of STAT3 that are induced in sequential fashion by all cytokines that utilize gp130 in all responding cells examined; these two forms of STAT3 apparently differ because of a time-dependent secondary serine/threonine phosphorylation involving an H7-sensitive kinase. In addition granulocyte colony-stimulating factor (GCSF), which utilizes a receptor highly related to gp130, also induces these two forms of STAT3. Furthermore, in contrast to a number of other cytokines and growth factors, all cytokines using gp130 and related signal transducers consistently and preferentially induce the two forms of STAT3 as compared with STAT1. While both tyrosine-phosphorylated forms of STAT3 bind DNA and translocate to the nucleus, the striking time-dependent progression from one form to the other implies important functional differences between the two forms. Our findings support the notion that differential acti-

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Abbreviations: STAT, signal transducer and activator of transcription; CNTF, ciliary neurotrophic factor; OSM, oncostatin M; LIF, leukemia-inhibitory factor; IL-6, interleukin 6; LIFR, leukemia-inhibitory factor receptor; IFN-α and IFN-γ, interferons α and γ; IL-3, interleukin 3; GCSF, granulocyte colony-stimulating factor; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride.

vation of individual STAT proteins by different classes of cytokines, coupled with secondary modifications of these STATs, could be involved in modulating the specificity of transcriptional responses.

MATERIALS AND METHODS

Cell Culture and Reagents. Cells (5) were serum-deprived for 2 to 4 hr prior to treatment with 50 ng of the indicated ligand per ml; in some cases cells were pretreated with 5 ng of interferon γ (IFN-γ) for 18–24 hr before serum deprivation. Human recombinant interferon α (IFN-α) was from Biosource International (Camarillo, CA); human recombinant GCSF, LIF, and OSM were from R & D Systems; biotinylated dUTP and IFN-γ were from Boehringer Mannheim; H7 was from Seikagaku America (Rockville, MD); poly(dI-dC)·poly(dI-dC) was from Pharmacia; STAT1 antibodies were from Transduction Laboratories (Lexington, KY); phosphotyrosine antibodies were from Upstate Biotechnology; and ImmunoPure immobilized streptavidin was from Pierce. STAT3 antibodies have previously been described (26).

Protein Precipitation. Phosphotyrosine immunoprecipitations and immunoblotting were done as described (15).

Oligonucleotide Synthesis and Affinity Reagents. The oligonucleotide pairs corresponding to response elements from c-*fos*/*sis*-inducible element [*m67* and *m34* (32) or *Ly-6E* gene (*GAS*; ref. 31)] were used to generate concatamers of binding sites for STAT91 and related DNA binding proteins. *m34* (5′-GTCGA CAGTT CACGT CAATC-3′, 5′-CGACG ATTGA CGTGA ACTGT-3′), *m67* (5′-GTCGA CATTT CCCGT AAATC-3′, 5′-CGACG ATTTA CGGGA AATGT-3′), or *GAS* (5′-GTCGT TATGC ATATT CCTGT AAGTG-3′, 5′-CGACC ACTTA CAGGA ATATG CATAA-3′) oligonucleotide pairs were phosphorylated, annealed, ligated, biotinylated, and then bound to streptavidin-agarose as described (31). Cytoplasmic extracts (≈2 mg/ml) were prepared as reported (31), stored at −80°C, and used as described (31). Briefly, cytoplasmic extracts were mixed with ≈24 pM agarose-bound oligonucleotide in the presence of 0.05% Triton X-100 and 75 μg of poly (dI-dC)·(dI-dC) per ml for 2 –4 hr at 4°C. The resulting pellet was washed twice with 1 ml of binding buffer without detergent. Bound proteins were eluted by using SDS protein sample buffer, subjected to SDS/PAGE on 7.5% acrylamide gels, and immunoblotted.

Gel Mobility-Shift Analysis. Analysis was performed as described (26).

RESULTS

p88/90 Undergoes a Time-Dependent Mobility Shift That Is Blocked by the Serine/Threonine Kinase Inhibitor H7. A detailed kinetic study of CNTF responses in EW-1 cells revealed that the relative amounts of p88 and p90 detected by phosphotyrosine immunoblotting vary in a reciprocal manner with time so that p90 appears as p88 disappears, suggesting that they might represent different forms of the same protein (Fig. 1*A Upper*). The apparent mobility shift from the 88-kDa form to the 90-kDa form displays a rather strict time dependence, initiating between 3 and 5 min after factor addition (Fig. 1*A*), and was observed in all cell lines tested and with all members of the CNTF family (e.g., Fig. 1 and data not shown).

Since the extent of tyrosine phosphorylation of the 88- and 90-kDa forms appeared to be similar as judged by phosphotyrosine immunoblotting, we reasoned that serine or threonine phosphorylation might instead be involved in the delayed mobility shift. To further examine this possibility, we used the serine/threonine kinase-specific inhibitor H7. H7 prevented the time-dependent shift in mobility of p88 to p90, without affecting the extent of tyrosine phosphorylation of p88 or of any other immediately induced tyrosine-phosphorylated proteins (Fig. 1*B Left* and data not shown); similarly, the omission of serine/threonine phosphatase inhibitors from the lysis buffer prevented observation of the shift, presumably because serine/threonine dephosphorylation of p90 occurred after lysis (data not shown). Altogether, our data suggest that the CNTF-related cytokines all induce immediate tyrosine phosphorylation of an 88-kDa protein, which then undergoes a mobility shift due to delayed serine/threonine phosphorylation.

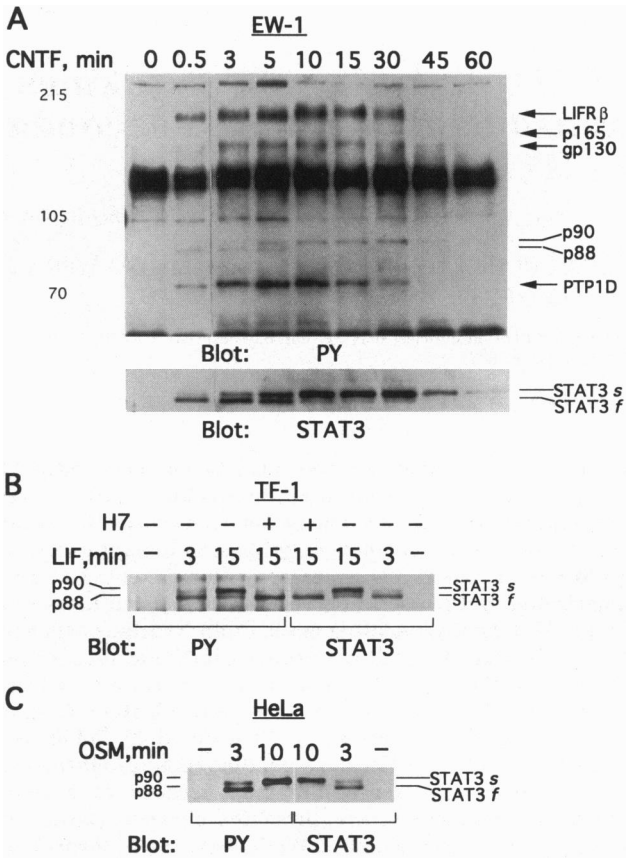

FIG. 1. CNTF induces rapid tyrosine phosphorylation and a time-dependent mobility shift of p88/p90, which correspond to sequentially activated forms of STAT3. (*A*) Extracts from EW-1 cells treated with CNTF for the indicated times were immunoprecipitated and then immunoblotted with phosphotyrosine-specific antibodies (*Upper*); the membrane was then stripped and reimmunoblotted with STAT3-specific antibodies (*Lower*). (*B*) Analysis as in *A*, except that extracts were prepared from TF-1 cells treated with LIF for the indicated times with or without pretreatment with 200 μM H7 for 30 min. (*C*) Analysis as in *A*, except that extracts were from HeLa cells treated with OSM for the indicated times.

p88 and p90 Correspond to Two Sequentially Activated Forms of STAT3. Cytokines of the CNTF family have been shown to activate both STAT1 and STAT3 (13, 15, 23, 26, 27). While p88 and p90 do not correspond to STAT1, they are weakly recognized by a subset of antibodies specific for STAT1, suggesting that they represent STAT-related proteins (13, 15, 23). We therefore immunoblotted the phosphotyrosine immunoprecipitates depicted in Fig. 1 with antisera specific for STAT3. This antiserum specifically and uniquely detected proteins precisely comigrating with p88 and p90 and exhibiting the same characteristic delayed mobility shift and sensitivity to H7 inhibition (Fig. 1*A Lower* and Fig. 1 *B* and *C Right*). These data indicate that p88 corresponds to a form of STAT3 that is rapidly tyrosine-phosphorylated in response to the CNTF family of cytokines and is thus designated STAT3f (for "faster" induction/migration) and that subsequently undergoes secondary serine/threonine phosphorylation to result in p90, a

more slowly migrating form of STAT3 hereon designated STAT3s (for "slower" induction/migration).

Both STAT3f and STAT3s Bind DNA and Translocate to the Nucleus. To determine whether both STAT3f and STAT3s could bind DNA, we examined their binding to the high-affinity c-*fos*/*sis*-inducible element (*m67*, see refs. 31 and 32) and the IFN-γ-activated sequence (*GAS*) from the *Ly-6E* gene (31), both of which are known to bind both STAT1 and STAT3; we also used a mutated version of the c-*fos*/*sis*-inducible element (*m34*; see ref. 32), which no longer binds STAT1, as a specificity control. Extracts from unstimulated and OSM-treated HeLa cells were incubated with agarose-coupled DNA recognition sequences, and proteins affinity-purified by binding to the DNA were detected by phosphotyrosine or STAT3-specific immunoblotting. Inducibly tyrosine-phosphorylated proteins that precisely comigrate with STAT3f and STAT3s and are recognized by STAT3-specific antisera could be affinity-purified by using the *m67* and *GAS* sequences but not the mutated *m34* element (Fig. 2*A*). Further confirming that the proteins bound to *m67* and *GAS* correspond to the previously defined STAT3f and STAT3s, the affinity-purified proteins exhibited the characteristic delayed mobility shift as well as the sensitivity of this shift to H7 (Fig. 2*B Upper* and Fig. 2*C*). Thus, both STAT3f and STAT3s can apparently bind to DNA, with both displaying similar sequence requirements as STAT1. Under these conditions we also affinity-purified low levels of STAT1 (Fig. 2*A Upper*).

The assays depicted in Fig. 2 *A–C* revealed which STAT proteins could be affinity-purified by using concatenated recognition sequences; they did not indicate whether the STAT proteins isolated could bind to single recognition sequences or whether they bound as homodimers or heterodimers. To address these issues, we performed gel mobility-shift analysis of nuclear extracts, using a radiolabeled oligonucleotide probe containing a high-affinity c-*fos*/*sis*-inducible element sequence (26). Extracts prepared from TF-1 cells stimulated with LIF, in which STAT1 activation is undetectable, were compared to extracts prepared from Hep G2 cells treated with IL-6 in which homodimeric complexes involving STAT1 (designated SIF-C) and STAT3 (designated SIF-A) as well as a heterodimeric complex (SIF-B) had previously been defined (26). This analysis revealed that two TF1 cell extracts containing predominately either STAT3f or STAT3s could form similar levels of SIF-A complexes (Fig. 2*D*). SIF-A composed of STAT3s migrated slightly faster on native gels (Fig. 2*D*, lane 3), presumably because of the increased negative charge of the additional serine/threonine phosphorylation. Altogether, the mobility-shift analysis reveals that both STAT3f and STAT3s are capable of forming SIF-A complexes involving homodimeric STAT3.

Secondary serine/threonine phosphorylation of STAT3 also does not appear to alter its ability to translocate into the nucleus, since both forms of STAT3 appeared in the cytoplasm and nucleus at similar levels and with similar time courses (Fig. 2*B Upper* vs. *Lower*).

Different Cytokines Selectively Activate Different Combinations of STAT Proteins. To compare the relative activation of different STATs by different families of cytokines and growth factors, we used HeLa cells that respond to OSM, a member of the CNTF family, and to IFN-α, IFN-γ, epidermal growth factor (EGF), and fibroblast growth factor (FGF). Both OSM and IFN-α predominantly induce STAT3 (since a 15-min time point is depicted, predominantly STAT3s is observed), while IFN-γ only induces the more slowly migrating STAT1 (Fig. 3*A*, lanes 4–7; STATs are distinguished by relative migration and immunoblotting with specific antisera). Like IFN-γ, both the epidermal and fibroblast growth factors induce only STAT1 in these cells, but much more weakly than the cytokines tested (Fig. 3*C*). Although both OSM and IFN-α induce STAT3, OSM induces significantly more STAT3. The

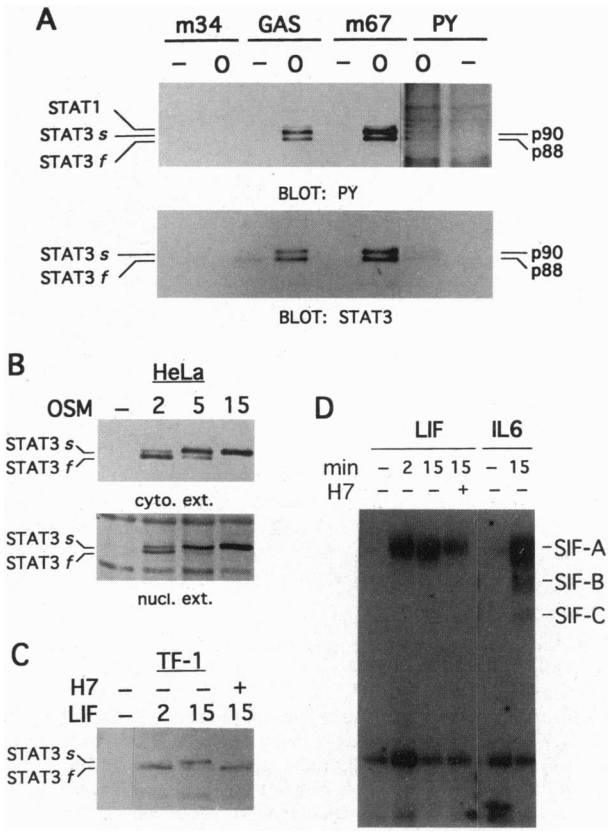

FIG. 2. Both STAT3f and STAT3s bind to the *GAS* and c-*fos*/*sis*-inducible DNA sequence elements and translocate to the nucleus. (*A*) Cytoplasmic extracts from HeLa cells were incubated with concatenated and agarose-bound *GAS*, *m67*, or *m34* DNA sequence elements or anti-phosphotyrosine agarose (PY), and affinity-purified proteins were then immunoblotted with phosphotyrosine-specific antibodies (*Upper*); the membrane was then stripped and reimmunoblotted with STAT3-specific antibodies (*Lower*). (*B*) Cytoplasmic (cyto.) (*Upper*) or nuclear (nucl.) (*Lower*) extracts (ext.) from HeLa cells treated with OSM for the indicated minutes were incubated with agarose-bound *m67*, and affinity-purified proteins were then immunoblotted with phosphotyrosine-specific antibodies. (*C*) Cytoplasmic extracts from TF-1 cells treated with LIF for the indicated minutes with or without 200 μM H7 pretreatment were incubated with agarose-bound *m67*, and affinity-purified proteins were then immunoblotted with phosphotyrosine-specific antibodies. (*D*) Gel mobility-shift analysis (with a radiolabeled oligonucleotide corresponding to the high-affinity c-*fos*/*sis*-inducible element *m67*) of nuclear extracts from TF-1 cells treated with LIF (with or without 200 μM H7 pretreatment) or Hep G2 cells treated with IL-6; complexes corresponding to SIF-A, SIF-B, and SIF-C are indicated.

preferential induction of STAT3 by OSM as compared with IFN-α is even more obvious in cells pretreated with IFN-γ. IFN-γ pretreatment increases the level of STAT1 phosphorylation (presumably because of the IFN-γ-dependent increased presence of STAT1 protein) but does not affect the level of STAT3 phosphorylation. Under these conditions OSM induces substantially more STAT3 binding activity and tyrosine phosphorylation as compared with those of STAT1, whereas the reverse is true for IFN-α (Fig. 3*A*, lanes 8–10). In addition, a longer exposure of the experiment depicted in Fig. 3*A* (lanes 8–10) revealed that IFN-α also induces STAT2, as previously shown, whereas OSM does not (Fig. 3*B*, lanes 1–3).

Because the GCSF receptor is homologous to gp130 and LIFR, we also examined GCSF responses for STAT3 induction. Since HeLa cells do not respond to GCSF, we used the NFS60 mouse myeloid leukemia cell line that responds to both GCSF and IL-3. In NFS60 cells we find that GCSF but not IL-3 induces the tyrosine phosphorylation and DNA-binding activ-

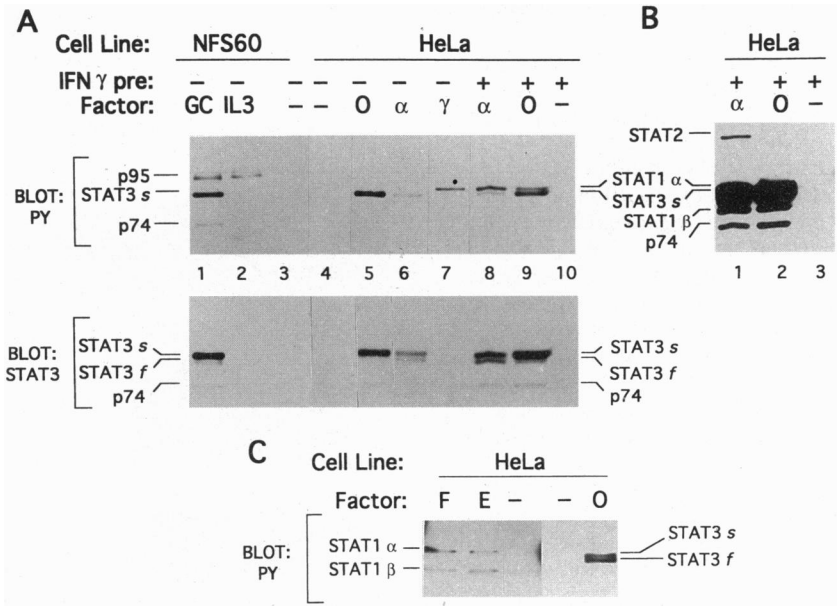

FIG. 3. Different cytokines selectively use different combinations of STAT proteins. (*A*) Cytoplasmic extracts (300 μg of total protein) from HeLa cells [pretreated (+) or not (−) with 5 ng of IFN-γ per ml for 20 hr prior to further stimulation] or NFS60 cells treated for 15 min with GCSF (lane GC), IL-3, OSM (lane O), IFN-α (lane α) or IFN-γ (lane γ) were incubated with the *m67* DNA sequence coupled to agarose, and the affinity-purified proteins were immunoblotted with phosphotyrosine antibodies (*Upper*); the membrane was then stripped and reimmunoblotted with STAT3-specific antibodies (*Lower*). Various STAT proteins are indicated; STAT3s but not STAT3f is seen because of the length of ligand exposure, and p95 and p74 potentially represent unrecognized STATs (see text). (*B*) Darker exposure of lanes 8–10 from *A Upper* show that STAT2 is specifically induced by IFN-α but not by OSM. Although it is not clear why STAT2 can be detected with our DNA affinity assay, since it has not been shown to bind the particular DNA sequence used, our assay may detect a weak affinity of STAT2 (perhaps in conjunction with STAT1) for this sequence. (*C*) HeLa cells were treated with epidermal growth factor (lane E), basic fibroblast growth factor (lane F), or OSM (lane O) to compare the level of STAT inductions; treated cells were analyzed as in *A Upper*, except that lanes 1–3 are from a 4-hr exposure whereas lanes 4 and 5 are from a 30-sec exposure.

ity of STAT3 (Fig. 3*A*, lanes 1–3). In addition, IL-3 and GCSF induce tyrosine phosphorylation of proteins of ≈95 and ≈74 kDa that also bind m67 (observed for IL-3 in longer exposures; data not shown), thus potentially representing additional STAT proteins; the 74-kDa protein is also weakly detected in OSM and IFN-α responses (see longer exposures in Fig. 3*B*) and reacts with STAT3-specific antisera (Fig. 3*A Lower*), suggesting that it may represent a lower molecular weight form of STAT3 previously described (26) or to a closely related STAT.

Altogether, these findings reveal that different factors can induce distinct combinations of STAT proteins. Interestingly, the level of STAT1 activation can be regulated by IFN-γ pretreatment, while activation of the related STAT3 is not altered by this pretreatment. Furthermore, the CNTF family of cytokines as well as GCSF, which utilizes a signal-transducing receptor component most homologous to those used by the CNTF family cytokines, seem to preferentially induce STAT3 as compared with STAT1.

DISCUSSION

Several major conclusions can be drawn from the data presented in this manuscript. (*i*) Cytokines that utilize gp130 and closely related signal transducers preferentially induce the tyrosine phophorylation of STAT3 relative to STAT1, consistent with other recent data (27, 30, 33). (*ii*) These cytokines sequentially activate two forms of STAT3: STAT3f, which is rapidly tyrosine-phosphorylated in response to these cytokines, followed by a more slowly migrating form, termed STAT3s. The prevention of the appearance of STAT3s by H7 suggests that STAT3s is formed by secondary serine/threonine phosphorylation of STAT3f involving an H7-sensitive kinase. (*iii*) Both STAT3f and STAT3s can bind DNA sequences normally used to evaluate STAT1 and STAT3 binding, and both forms undergo nuclear translocation.

The functional role of the delayed serine/threonine phosphorylation that causes the shift of STAT3f into STAT3s remains a mystery. It is quite possible that the secondary phosphorylation in some way directly modulates the DNA binding specificity of STAT3 in a subtle way not revealed by our analysis of a limited number of DNA sequences. Alternatively, this phosphorylation may alter the ability of STAT3 to participate in the activation of gene transcription by affecting its interactions with other STATs or accessory proteins it associates with to activate transcription. Consistent with this possibility, H7, which blocks these serine/threonine phosphorylations, is known to block transcriptional gene responses induced by both IFN-γ (34) and the CNTF family of cytokines (5, 35, 36). Interestingly, H7 also blocks activation of extracellular signal-regulated protein kinase (ERK)/mitogen-activated protein (MAP) kinases by the CNTF-related cytokines, but not that by other classes of cytokines and growth factors (unpublished data, T.G.B., N.S., and G.D.Y.). ERK/MAP kinase activation by the CNTF-related cytokines occurs concurrently with the STAT3 shift and, thus, may involve the same H7-sensitive kinase (unpublished data, T.G.B., N.S., and G.D.Y.). This possibility may provide the first link between two pathways, one involving the ERK/MAP kinases and the other involving the STATs, previously thought to provide independent modes of signaling to the nucleus; the ability of H7 to block gene responses may thus involve both these pathways. In addition to the possibilities considered above, it is conceivable that the secondary phosphorylations of STAT3 are somehow involved in the regulation/deactivation of this transcriptional activator. Regardless, the striking and regulated nature of the STAT3f/STAT3s progression demands further exploration of the functional role of serine/threonine

phosphorylation on the STATs in general, and STAT3 in particular.

Our data extend previous findings that different cytokines can differentially activate distinct combinations of STAT proteins. While STAT1 appears to be activated by a wide variety of growth factors and cytokines (31, 37–41) and STAT2 appears to be rather specific to IFN-α (24, 25), STAT3 is preferentially activated by the CNTF family of cytokines or by GCSF (27, 30, 33), all of which use related signal-transducing receptor components. Thus, STAT3 activation can result from gp130 homodimerization (as induced by IL-6), gp130/LIFR heterodimerization (as induced by CNTF, LIF, and OSM), heterodimerization of gp130 with the alternative OSM signal transducer [as occasionally induced by OSM (11)], or homodimerization of the GCSF receptor. What then determines whether or not a particular receptor complex induces a given STAT? While early data suggested that this specificity might be governed by the particular Jak/Tyk kinase activated by a given receptor complex (14, 19, 42), recent findings argue that the choice of STATs and other substrates acted on by the Jak/Tyk kinases is instead specified by modular tyrosine-based motifs in the signal-transducing receptor components (7, 12, 21, 22, 43–45).

Just as it is possible that STAT3f and STAT3s act on different targets *in vivo* despite their similar sequence requirements *in vitro*, different STATs may also act on distinct target genes *in vivo*; differences in the accessory proteins they interact with [such as p48/interferon-stimulated gene factor 3 (ISGF3γ); see refs. 24 and 25], heterodimerization between related STATs or secondary modifications of STATs such as serine/threonine phosphorylations may be involved in varying target-gene specificity. These possibilities may explain the existence of several different STAT proteins with seemingly similar DNA-binding specificities *in vitro*, many simultaneously activated by the same factor. In addition, the ability of a STAT protein to access particular target genes may depend on the cell in which it is activated. The same target gene may be in an "accessible" chromatin configuration in one cell but not in another. These considerations possibly explain how STAT3 may be involved in regulating acute-phase response genes in a liver cell (in response to LIF or IL-6) but a totally different set of genes in a neuron (e.g., that encoding a neuronal marker such as vasointestinal peptide in response to CNTF) or a hemopoietic cell (in response to IL-6).

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