Epidermal growth factor induces the tyrosine phosphorylation and nuclear translocation of Stat 5 in mouse liver

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Contributed by Stanley Cohen, January 5, 1995

ABSTRACT
Intraperitoneal injection of epidermal growth factor into mice results in the appearance of multiple tyrosine-phosphorylated proteins in liver nuclei within minutes after administration. We have previously identified three of these proteins as Stat 1α, Stat 1β (p91, p84), and Stat 3 (p89). In the present report we demonstrate that Stat 5 (p92), the recently described prolactin inducible transcription factor detected in mammary glands, is the major tyrosine-phosphorylated protein translocated to the nucleus in mouse liver in response to epidermal growth factor. Furthermore, gel-shift analysis and affinity purification revealed that Stat 5, Stat 1α, and Stat 1β specifically bind to the prolactin inducible element upstream of the β-casein promoter.

Epidermal growth factor (EGF) elicits a variety of biological responses when administered either to intact animals or added to cells growing in culture (1–3). These responses are mediated at the cell surface by the EGF receptor. Upon ligand binding, the intrinsic tyrosine kinase activity of the EGF receptor is augmented, resulting in its autophosphorylation, as well as the phosphorylation, of specific intracellular protein substrates (4–6). Some of these tyrosine-phosphorylated proteins are responsible for initiating signal transduction from the cell membrane to the nucleus and have been termed Stat (signal transducers and activators of transcription) proteins (7).

The Stat proteins are members of a growing family of transcription factors that reside in the cytosol, are activated by tyrosine phosphorylation, and are translocated to the nucleus in response to various growth factors and cytokines. Stat 1α/1β (p91, p84) was shown to be tyrosine-phosphorylated and form specific binding complexes with the sis-conditioned medium inducible element (SIE) upstream of the c-fos promoter in response to both interferon γ and EGF (8, 9). Similarly, Stat 3 (p89) was shown to be tyrosine-phosphorylated and bind the SIE in response to both EGF and interleukin 6 or lipopolysaccharide (10, 11). Recently, several reports have appeared that describe the induction in sheep mammary glands of DNA-binding activity specific for a prolactin (PRL)-inducible element (PIE). This PRL-induced DNA-binding activity was attributed to a 92-kDa protein that has been termed Stat 5 (or mammary gland factor) due to sequence homologies with conserved regions of known Stat proteins (12, 13).

We previously reported that injection of EGF into mice leads to a rapid increase in the level of tyrosine phosphorylation of many proteins in all organs examined (14). Using this in situ system we were able to detect at least four tyrosine-phosphorylated proteins (p92, p91, p89, and p84) in nuclear extracts from the livers of mice treated with EGF. Three of these proteins, p91, p89, and p84, were identified as Stat 1α, Stat 3, and Stat 1β, respectively (8, 11). However, the identity of the major tyrosine-phosphorylated protein detected in the nucleus in response to EGF, p92, was unknown. In this report, we identify p92 as Stat 5.

MATERIALS AND METHODS
Materials. ND4 Swiss–Webster mice were obtained from Harlan–Sprague–Dawley. EGF was prepared as described (14). Immobilon-P membranes were from Millipore. RC20H (horseradish peroxidase-conjugated recombinant antibody fragment specific for phosphotyrosine), anti-Stat 1α,β [anti-interferon stimulated gene factor 3 (ISGF3) monoclonal and polyclonal antibody], and anti-Stat 5 monoclonal antibody were from Transduction Laboratories (Lexington, KY). Polyclonal anti-Stat 3c was prepared as described (10). Goat anti-mouse IgG labeled with horseradish peroxidase was from Cappel Laboratories. Enhanced chemiluminescence reagent was from Amersham. Prestained molecular weight standards were from Life Technologies (Gaithersburg, MD). Avidin–agarose was from Pierce. All oligonucleotides were from Oligo Inc. (Wilsonville, OR). Poly(dI-dC)-poly(dI-dC) was from Pharmacia Biotech. All other reagents were from Sigma.

Preparation of Nuclear Extracts. Solutions of EGF (1 mg/ml) or phosphate-buffered saline (PBS) were injected i.p. into mice at a dose of 10 μl/g of body weight. Mice were sacrificed by cervical dislocation 15 min after injection, and the livers were removed and immediately frozen in liquid nitrogen. Mouse liver nuclei were isolated, purified by centrifugation through a 2.2 M sucrose cushion, and extracted with 0.2 M sodium chloride as described (8).

Mobility-Shift and Supershift Analyses. The sequence of the double-stranded PIE used in the gel shift was 5′-GGA CTT GGA ATT AAG GGA-3′. The sequences of the SIE, guanylate-binding protein (GBP), and interferon α/β-stimulated response element (ISRE) were described (8). All incubations were done at room temperature. Portions (5 μl) of nuclear extracts from control or EGF-treated animals were incubated with 2 μg of poly(dI-dC)-poly(dI-dC) in 3 mM EDTA/1 mM dithiothreitol/4% (wt/vol) Ficoll containing an excess of unlabeled oligonucleotide, where indicated, in a final volume of 20 μl for 10 min. 32P-labeled oligonucleotide (1 × 108 cpn, ∼1 ng), prepared as described (8), was then added to the mixture, and incubation was continued for 15 min. Supershift analysis of SIE- and PIE-binding proteins was done by including either 5 μl of polyclonal anti-Stat 1 or 1 μl of polyclonal anti-Stat 3c or 2 μl of monoclonal anti-Stat 5 (as indicated) in the first 10-min incubation period.

Affinity Purification and Identification of PIE-Binding Proteins. Solutions of EGF (1 mg/ml) or PBS were injected i.p. into mice at a dose of 10 μl/g of body weight, and the livers were homogenized (25% wt/wt) in buffer A (20 mM Heps, pH 7.9/0.1 M sodium chloride/1 mM sodium orthovanadate/50 mM β-glycerol phosphate/10 mM sodium fluoride/50 μM sodium molybdate). The homogenate was centrifuged at 100,000 × g for 1 hr. The resulting supernatant was supplemented with glycerol to 10%, dithiothreitol to 1%

Abbreviations: EGF, epidermal growth factor; SIE, sis-conditioned medium inducible element; PRL, prolactin; PIE, prolactin-inducible element; GBP, guanylate-binding protein; ISRE, interferon α/β-stimulated response element.

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mM, 10 μg of poly(dl-dC)-poly(dl-dC), and 10 μg of competing oligonucleotide (where indicated) in a final volume of 1 ml and incubated at 4°C for 30 min. Biotinylated–PIE oligonucleotide (2 μg) was added and incubated for 2 hr followed by the addition of 50 μl (50% slurry) of avidin–agarose for 30 min. The suspensions were centrifuged, and the sedimented material was washed five times with buffer A supplemented with glycerol to 10%. The bound proteins were eluted by boiling in 2× Laemmli buffer (50 μl) (15) for 5 min, resolved by SDS/PAGE (7%), transferred to Immobilon, and immunoblotted with an antibody to phosphotyrosine. Proteins were detected with enhanced chemiluminescence reagent (ECL). The blot was stripped (8) and reprobed with a 1:1000 dilution of monoclonal anti-Stat 1α/β for 1 hr, followed by a 1:1500 dilution of goat anti-mouse IgG labeled with horseradish peroxidase for 1 hr. Antibody binding was detected with ECL. The same blot was again stripped and reprobed with a 1:500 dilution of monoclonal anti-Stat 5 followed by secondary antibody incubation and ECL detection as described above.

RESULTS AND DISCUSSION

Detection of PIE-Binding Complexes in Nuclear Extracts from the Livers of EGF-Treated Mice. Nuclear extracts from the livers of control and EGF-treated mice were analyzed for the ability to form specific PIE-binding complexes in a gel-shift assay. Proteins in nuclear extracts from control animals had no detectable PIE-binding activity. However, two protein–PIE binding complexes (labeled A and B) could be detected in nuclear extracts from EGF-treated animals (Fig. 1A). Both PIE–A and PIE–B complexes could be completely disrupted by a 100-fold excess of either unlabeled PIE or SIE. However, PIE–B appeared to have a greater affinity for SIE than PIE–A: the PIE–B complex could be disrupted by a 10-fold excess of SIE, whereas the PIE–A complex required >50-fold excess of SIE for complete disruption (Fig. 1B). In addition, as shown in Fig. 1A, only the PIE–B complex could be disrupted by a 100-fold excess of unlabeled GBP (the interferon γ-inducible element). The addition of a 100-fold excess of unlabeled ISRE had no effect on the appearance of either the PIE–A or PIE–B complexes. These experiments suggested that PIE–B may be related to Stat 1α/1β, a previously identified SIE-binding protein and that PIE–A is an unknown EGF-induced DNA-binding protein.

Supershift Analyses of SIE- and PIE-Binding Complexes. To directly determine whether the EGF-induced PIE-binding complexes contained either Stat 1 or Stat 3 we performed supershift analyses with antibodies specific for each of these proteins and compared those results with results obtained by using SIE as the probe (Fig. 1C). Little SIE- or PIE-specific binding was detectable in nuclear extracts from control animals. As previously reported, nuclear extracts from livers of EGF-treated mice contain three specific SIE-binding complexes (SIE-A, -B, and -C) (8, 11) and, as shown in this report, two specific PIE-binding complexes (PIE-A and -B). Antibodies specific for Stat 3 supershifted the upper two SIE complexes (A and B) from EGF-treated extracts as previously reported (11) but had no effect on the migration of the PIE-binding complexes (A or B) or of the SIE–C complex. Antibodies specific for Stat 1α/1β supershifted both SIE–C and PIE–B in the gel-shift reactions but had no effect on PIE–A. Consistent with these results, the SIE–C complex was effectively inhibited by the addition of excess unlabeled PIE (data not shown). From these experiments we conclude that (i) Stat 1α/1β is a component of both SIE–C and PIE–B, (ii) Stat 3 is a component of SIE–A, and (iii) PIE–A is generated by a protein that is immunologically distinct from Stat 1 and Stat 3.

Affinity Purification of PIE-Binding Proteins. Because the recently cloned Stat 5 protein is known to bind the PIE element in response to PRL and the protein responsible for the EGF-induced PIE–A appeared different from the known SIE-binding proteins, we affinity-purified the PIE-binding proteins to determine whether Stat 5 was activated in response to EGF. To isolate PIE-binding proteins, cytosolic extracts from livers of control animals and livers from animals treated with EGF for 15 min were incubated with the nonspecific competitor poly(dl-dC)poly(dl-dC) alone or poly(dl-dC)poly(dl-dC) plus excess competing oligonucleotide and then incubated with biotinylated PIE and avidin–agarose. The adsorbed proteins were eluted and separated by SDS/PAGE and analyzed by immunoblotting with monoclonal antibodies to phosphotyrosine, Stat 1, and Stat 5 (Fig. 2). No tyrosine-phosphorylated proteins were detected on the biotinylated–PIE–avidin–agarose beads after incubation with liver extracts from control animals. Two major tyrosine-phosphorylated bands of ~92 and 90 kDa and one very minor

![Fig. 1](image-url)
band of 84 kDa were present among the proteins adsorbed by biotin–PIE in the cytosol from EGF-treated animals (Fig. 2, lane 2). Adding an excess of ISRE to the adsorption reaction had no effect on the appearance of these bands (lane 4). However, adding an excess of GBP resulted in the disappearance of pp84 (lane 3). Reprobing the blot with anti-Stat 1α/1β revealed that Stat 1α and Stat 1β were bound to the PIE-labeled agarose beads after treatment with EGF and that excess ISRE had no effect on this binding (lanes 6 and 8). However, the binding of Stat 1α/1β to PIE could be effectively disrupted by adding excess GBP to the adsorption reaction (lane 7). Reprobing of the blot with anti-Stat 5 revealed that Stat 5 possessed PIE-binding activity in response to EGF treatment and that this binding could not be disrupted by excess of either ISRE or GBP (lanes 9–12). These results are in agreement with the data reported in Figs. 1A and C. From these experiments we conclude that both Stat 1α/1β and Stat 5 are tyrosine-phosphorylated and capable of binding PIE in response to EGF. The appearance of Stat 5 as a doublet (90–92 kDa) is probably due to extents of phosphorylation.

**EGF Induces the Nuclear Translocation of Stat 5.** To determine whether any of the PIE-complexes detected in nuclear extracts from livers of EGF-treated animals (see Fig. 1) contain Stat 5, we performed supershift analyses of both the PIE- and SIE-binding complexes with an antibody to Stat 5 (Fig. 3). Antibodies specific for Stat 5 had no effect on any of the observed SIE-binding complexes or the PIE–B complex. However, this antibody disrupted the PIE–A complex. From this experiment we conclude that Stat 5 is a component of the EGF-induced PIE–A complex.

We next examined nuclear extracts from livers of control mice and mice treated with EGF for the presence of phosphotyrosine, Stat 5, Stat 3, and Stat 1α/1β by immunoblotting with the appropriate antibody (Fig. 4). As reported previously, no tyrosine-phosphorylated proteins were seen in nuclear extracts from control animals. However, after EGF treatment several tyrosine-phosphorylated proteins, ranging in size from 84 to 92 kDa, were detected (8). Reprobing the same blot with monoclonal anti-Stat 5 revealed the presence of the 92-kDa Stat 5 protein, but only in the nuclear extracts from EGF-treated animals. Consistent with previous results, reprobing the blot with monoclonal antibodies to Stat 1α/1β and Stat 3 revealed the presence of these proteins in the nucleus, but, again, only after EGF treatment (8, 11). The series of immunoblots illustrated in Fig. 4 also demonstrates the relative migrations of these EGF-induced Stat proteins when examined by SDS/PAGE.

Neither tyrosine phosphorylation of Stat 5 nor PIE-binding activity was detected in the livers of PRL-injected mice (data not shown). Therefore, we were unable to directly compare the effects of EGF and PRL treatment on the extent of Stat 5 tyrosine phosphorylation in mouse liver.

Although the PIE element was derived from a region upstream of the β-casein promoter, we do not know the identities of genes that contain the PIE element or related elements whose transcription is altered by EGF in the liver.

We conclude that (i) Stat 5 is the major tyrosine-phosphorylated protein translocated to the nucleus in the livers of EGF-treated mice, (ii) two specific PIE-binding complexes (designated PIE–A and PIE–B) are present in liver nuclear extracts from EGF-treated mice, (iii) Stat 1 is a component of the PIE–B complex and Stat 5 is a component of the PIE–A complex, (iv) both PIE–A and PIE–B binding proteins can interact with the SIE element but the latter can interact with a greater apparent affinity, and (v) Stat 5 does not heterodimerize with Stat 1α/1β in response to EGF.

We thank Dr. James Darnell, Jr. (The Rockefeller University), for Stat 3c polyclonal antibodies. This work was supported by Public Health Service Grant HD-00700 and Training Grant CA-09582.