Growth Hormone-induced Signal Transduction Depends on an Intact Ubiquitin System*

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The growth hormone receptor (GHR) is a ubiquitinated cell surface protein. Ligand binding and receptor dimerization activate the cytosolic kinase Jak2. This event initiates signal transduction via STAT proteins. Expression of GHR in a Chinese hamster ovary (CHO) cell line, which exhibits a temperature-sensitive defect in ubiquitin conjugation (CHO-ts20), as well as in wild type cells (CHO-E36) has shown that endocytosis of the receptor requires an intact ubiquitin conjugation system (Strous G. J., van Kerkhof, P., Govers, R., Ciechanover A., and Schwartz, A. L. (1996) EMBO J. 15, 3806–3812). We have now examined the requirement for ubiquitin conjugation in growth factor-mediated signal transduction. In CHO-E36 and in CHO-ts20 cells at the permissive temperature, STAT proteins were activated in a growth factor-dependent fashion. However, no activation of STAT proteins was observed at the nonpermissive temperature in CHO-ts20 cells. Neither tyrosine phosphorylation of GHR nor of Jak2 was inhibited at the nonpermissive temperature. When tyrosine phosphorylation was inhibited following treatment with staurosporin, ubiquitination of the receptor proceeded normally. Furthermore, mutation of GHR phenylalanine-327, which prevents GHR endocytosis, inhibited receptor ubiquitination but allowed normal Jak/STAT-mediated signal transduction. Thus, these data provide evidence that the ubiquitin conjugation system is involved in the Jak/STAT signaling pathway, be it at the initial stage(s) of Jak2 activity.

Binding of growth hormone (GH)1 to its receptor causes dimerization of two growth hormone receptor (GHR) polypeptides, which in turn initiates a cascade of events leading to signal transduction in the cell nucleus and down-regulation of the receptor (1, 2). Lacking intrinsic tyrosine kinase activity, the GHR recruits and activates a member of the Janus family of cytosolic kinases (Jak2) upon dimerization (3). In addition to the GHR polypeptides and itself, Jak2 phosphorylates special signal transducers and activators of transcription proteins (STATs), which translocate to the nucleus and convey the appropriate signal to specific regulatory DNA-responsive elements (5–8).

The ubiquitin system has been established as the major regulatory protein degradation system within eukaryotic cells (9–12). Proteins destined for degradation are covalently modified by conjugation of multiple ubiquitin polypeptides following recognition and modification by members of the E2 (ubiquitin conjugating) and E3 (ligating) protein families. Recently, the ubiquitin conjugation system has been shown to play a role in other regulatory functions such as control of protein kinase activity (13) and receptor endocytosis (14, 15). An intact ubiquitin conjugation system is required for prompt ligand-induced GHR endocytosis and degradation (15). Many other cell surface receptors are ubiquitinated upon ligand binding (16–20), although their dependence upon ubiquitin conjugation for endocytosis has not been addressed. As signal transduction and down-regulation of most cell surface signaling receptors are tightly coupled, we have examined the role of the ubiquitin conjugation system in GHR signal transduction.

We have utilized GHR-transfected CHO-ts20 cells that express a thermolabile ubiquitin activating enzyme, E1 (21). At the nonpermissive temperature ligand-induced endocytosis of the GHR is blocked in these cells (15). Herein, we report that although GH-induced tyrosine phosphorylation of GHR and Jak2 at the cell surface is not affected at the nonpermissive temperature, the Jak2/STAT signaling pathway is completely inhibited.

MATERIALS AND METHODS

Cells and Materials—Cell lines derived from CHO-ts20 and CHO-E36 cells, stably transfected with rabbit GHR-cDNA, were used (15). The GHR-F327A mutant was prepared by inserting a polymerase chain reaction product, using a 5′-oligonucleotide, containing a PstI site (GATCCCCACCCATTGGCCTCAACTGGACTTT) and a 3′-oligonucleotide containing a ClaI site and a TTC → GCC mutation (F → A) (GATCCATGATGCTACGTCGATGCTCGTCA). The DNA sequence was confirmed for the entire polymerase chain reaction fragment. Cells were grown in Eagle's minimal essential medium supplemented with 4.5 g/liter glucose, 10% fetal bovine serum, penicillin, and streptomycin, and 0.4 mg/ml genetin. For experiments the cells were grown on 35-mm plates and used at 75% confluence. The CHO-ts20 clone used in this study expressed approximately 10-fold more receptor than the CHO-E36 clone. To compensate for this, 10 mM sodium butyrate was added to CHO-E36 cells 18 h before use, increasing GHR expression approximately 5-fold (15). As before, the sodium butyrate treatment did not alter the behavior of GHR in any of the parameters examined in this study. Anti-GHR antiserum was raised in rabbits against a fusion protein of glutathione S-transferase and a GHR tail peptide consisting of amino acids 327–493 (15). The fusion protein was expressed in Escherichia coli after transformation with the appropriate pGEX-2T DNA construct (Pharmacia Biotech Inc.). Anti-PY and rabbit anti-mouse STAT3 were from Upstate Biotechnology, Inc. (Lake Placid, NY), and anti-Jak2 (monoclonal antibody against the N-terminal 194 amino acid sequence of STAT1) from Transduction Labs (Lexington, KY). Antiserum against Jak2 was raised in rabbits against a synthetic peptide corresponding to the hinge region between domains 1 and 2 of murine Jak2. Antiserum specific for ubiquitin-protein conjugates was a

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1 The abbreviations used are: GH, growth hormone; GHR, growth hormone receptor; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; PY, phosphotyrosine; SIE, c-sis-inducible element.

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The gap was filled using the Klenow reaction in the presence of aprotinin, and 1 mM phenylmethylsulfonyl fluoride in PBS. The reactions were incubated for 2 h at 0 °C with 5 μl of specific rabbit anti-GHR antisera; protein A-agarose (Repligen Co., Cambridge, MA) was used to isolate the immune complexes. The immunoprecipitates were washed twice with the same buffer and once with 10-fold diluted PBS; immune complexes were analyzed by 7.5% polyacrylamide gel electrophoresis in the presence of SDS.

After transfer to polyvinylidene difluoride paper, the blots were immunostained using anti-ubiquitin conjugate antibody, anti-GHR (after stripping of the blots), and anti-PY (15). The antigens were visualized using the ECL system (Amersham Corp.).

Transcription Factor Complexation Assays—Either total cell lysates or nuclear extracts from 2 × 10^6 cells were incubated with a ^32P-labeled oligonucleotide known as the c-sis-inducible element (SIE) of the c-fos gene promoter as described (22, 23). To prepare radiolabeled SIE two oligonucleotides were synthesized (5'-GTCGACATTTCCCGTA-AATCGTCGA-3' and 5'-TCGACGATTACG-3') and hybridized, and the gap was filled using the Klenow reaction in the presence of [α-32P]ATP and unlabeled dCTP, dGTP, and dTTP. Transcription factor complex formation and electrophoresis were performed as described (22).

RESULTS

In order to determine whether the ubiquitin conjugation system is involved in signal transduction of the GHR, CHO-ts20, and CHO-E36 cells stably transfected with GHR were incubated at the permissive (30 °C) and nonpermissive (42 °C) temperatures, and the cell and nuclear extracts were examined for GH-inducible band shifts using ^32P-labeled SIE. As seen in Fig. 1A, GH induced a clear signal in CHO-E36 cells at both the permissive and nonpermissive temperatures and in CHO-ts20 cells incubated at the permissive temperature. Closer examination of the band pattern (Fig. 1B) reveals that the specific signal is composed of three bands (a, b, and c). If excess unlabeled SIE was added, all three bands disappeared (Fig. 1B, lanes 2 and 3); if antibodies against STAT1 were present the STAT complex formation in CHO cells. These observations together with data from literature indicate that the three-band pattern represents homo- and heterodimers of STAT1 and STAT3 (5). In nuclear extracts from CHO-E36 cells the lower band (Fig. 1B, c) was lacking (Fig. 1A).

If CHO-ts20 cells were incubated at the nonpermissive temperature in the presence of GH, no STAT complexes were detectable. This observation was consistently made over a temperature range of 40–42 °C, and the STAT complex formation coincided fully with the capacity of the CHO-ts20 cells to activate ubiquitin. The weak signal at lower electrophoretic mobility in this lane did not disappear if the incubation was carried out in the presence of excess unlabeled probe. If CHO-ts20 cells were used transfected with a mutant DNA (GHR-F327A), the same results were obtained as with wild type GHR. No STAT complex was observed in these cells following incubation at the nonpermissive temperature in the presence of GH (Fig. 1C). Together, the data show that an intact ubiquitin conjugation system is necessary for GH-mediated Jak/STAT signaling.

The F327A mutation in the GHR was previously shown to affect ligand-induced endocytosis, not signal transduction (24). As seen in Fig. 1C, CHO-ts20 cells transfected with GHR-F327A DNA show normal signaling via STAT pathway upon GH addition at the permissive temperature. The mutated GHR in these cells is not ubiquitinated upon GH addition at the permissive temperature. Thus, although endocytosis and ubiquitination of the GHR were inhibited in the GHR-F327A mutant, Jak/STAT signal transduction occurred normally. This result also shows that ubiquitination of the receptor tail is not required for signal transduction.

Following dimerization of the GHR polypeptides, tyrosine phosphorylation of both GHR and Jak2 is the initial event in the signaling cascade. Therefore, we examined whether an intact ubiquitin conjugation system is essential for this event. In all cases a substantial phosphotyrosine signal was observed

FIG. 1. Protein complex formation with ^32P-labeled DNA SIE element. The binding of nuclear (A) and whole cell extracts (C) was performed as described under "Materials and Methods." Cells were cultured in serum-free medium overnight, and the incubation was continued at the temperatures indicated in Eagle's minimal essential medium supplemented with 20 mM Hepes, pH 7.2, for 60 min, followed by a 15-min incubation in the presence or the absence of 8 nM GH. Cellular or nuclear extracts were analyzed in the transcription factor complexation assay, and the label patterns were visualized by a PhosphorImager (Molecular Dynamic). A and C represent different experiments. In B, the specificity of the reaction was determined using the same extract as in the second lane of A (CHO-ts20, incubated at 30 °C for 15 min in the presence of GH); additions to the complex formation: lane 1, none; lane 2, 100 times excess unlabeled probe; lane 3, 10 times excess unlabeled probe; lane 4, 0.5 μg of anti-STAT3; lane 5, 0.12 μg of anti-ISGF3. Due to relatively high protein concentrations in the total cell lysates (C), the specific complexes (arrowhead) migrate as diffuse bands as compared with the nuclear extracts (A). The lower intense band in all lanes of C are nonspecific, because they do not disappear in the presence of excess unlabeled probe.

2 R. Govers and G. J. Strous, unpublished observation.
on both the mature GHR and Jak2 upon the addition of GH. In the case of Jak2 (Fig. 2, right panel) an extra diffuse and slower migrating PY-labeled band is visible, most probably originating from co-immunoprecipitation of GHR. A somewhat diminished PY signal was observed in both GHR and Jak2 in CHO-ts20 and in CHO-E36 cells following incubation at temperatures above 40°C. Occasionally the decrease in signal was more prominent in the CHO-ts20 than in CHO-E36 cells. This variability is likely due to temperature-dependent activity of Jak2. Thus, it is unlikely that the ubiquitin conjugating system is required for Jak2 recruitment nor for its activation. We also examined the extent to which the GHR-F327A mutant was phosphorylated in response to ligand. Both receptor and Jak2 phosphorylation were similar to that observed with the wild type receptor (not shown).

Because both GHR tyrosine phosphorylation and ubiquitination appear to occur at the cell surface, it is important to determine whether the two events are independent. Thus, to eliminate indirect effects of phosphorylation on receptor ubiquitination, we utilized the phosphokinase inhibitor staurosporin. As seen in Fig. 3, staurosporin inhibited the ligand-induced GHR phosphorylation almost completely. However, the GH stimulation of GHR ubiquitination was unaffected. Of note is that no PY signal is visible at the position of ubiquitinated GHR (Fig. 3, middle panel), indicating that ubiquitinated GHR is not phosphorylated. Taken together with the results described above, we conclude that ubiquitin conjugation and tyrosine phosphorylation of the GHR (and Jak2) are independent events.

**DISCUSSION**

The ubiquitin conjugating system plays a pivotal role in a diverse array of regulatory events including cell cycle progression, DNA repair, and transcriptional control. Many of these processes are dependent upon both ubiquitin conjugation to target proteins as well as the subsequent degradation of the protein-ubiquitin moieties via the proteasome. In the present study we demonstrate that the ubiquitin conjugating system is involved in signal transduction via the Jak/STAT pathway. This pathway mediates transduction of a wide variety of extracellular signals via receptors for interferon, many cytokines, and GH (3–5, 7, 8, 25). Using cells that display an intact GH-GHR pathway yet contain a temperature-sensitive mutation in the initial enzyme of the ubiquitin conjugation pathway, we have demonstrated that inactivation of the ubiquitin pathway inhibits GH-induced stimulation the Jak/STAT pathway.

Our data do not define at which stage(s) ubiquitin conjugation is involved in the signaling cascade, although the data render it unlikely that the initial steps that likely occur at the cell surface (receptor dimerization, Jak2 recruitment, and Jak2 activation) are affected by the ubiquitin system. Previously, we have shown that the ubiquitin system is involved in receptor endocytosis (15). These results together with the current data suggest that the cell surface is the likely site of GHR signaling. Dependent upon the GH concentration, temperature, and cell type, ligand-induced endocytosis of the GHR occurs with a half-time of 30–60 min (26–28). Tyrosine phosphorylation, however, occurs maximally within 15–20 min (Ref. 29 and Fig. 2). Because no photophosphorylase-labeled GHR was found to be ubiquitinated and because the two events appear to be independent, it is tempting to speculate that phosphorylation and subsequently Jak/STAT transduction occur first, following which the receptor is subject to endocytosis mediated by the ubiquitin conjugation system.

As proposed for the role of ubiquitination in ligand-induced endocytosis of the GHR, ubiquitination in addition to protein phosphorylation may act as a control point in regulating some step(s) in the Jak/STAT signaling cascade. However, because many basic cellular processes are regulated subsequent to ubiquitin conjugation, use of the E1 mutant cell line at the nonpermissive temperature does not exclude the possibility that other E1-dependent events are critical in the control of this signaling pathway.

Recently, Allevato and colleagues (24) have reported that phenylalanine 346 within the rat GHR cytoplasmic tail is critical for ligand-induced internalization but not essential for transcriptional control. We have made the analogous mutation in the rabbit receptor, which results in defective endocytosis as well as ubiquitination.2 These results further support the notion that the ubiquitin conjugating system acts on the signaling pathway downstream from the tyrosine phosphorylating action of Jak2. Recently, Chen et al. (13) have shown that the protein kinase involved in the activation of the transcription factor NFκB depends on the presence and function of several factors of the ubiquitin conjugation system. Thus, the ubiquitin system appears to be involved in a variety of growth control and signal transduction pathways. Future studies will focus on the mechanism(s) involved in modulation of the Jak/STAT pathway by the ubiquitin system.

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