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Calmodulin-dependent protein kinases phosphorylate gp130 at the serine-based dileucine internalization motif

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Abstract

The receptor for leukemia inhibitory factor (LIF) consists of two polypeptides, the low affinity LIF receptor (LIFR) and gp130. We previously demonstrated that LIF stimulation caused phosphorylation of gp130 at Ser782, adjacent to a dileucine internalization motif, and that transient expression of a mutant receptor lacking Ser782 resulted in increased cell surface expression and increased LIF-stimulated gene expression compared to wild-type receptor. Phosphorylation of Ser782 on gp130 fusion protein by LIF-stimulated 3T3-L1 cell extracts was inhibited 61% by autocamtide-2-related inhibitory peptide (AIP), a highly specific and highly effective inhibitor of calmodulin-dependent protein kinase type II (CaMKII). Purified rat forebrain CaMKII was also able to phosphorylate gp130 fusion protein at Ser782 in vitro. Furthermore, antibodies targeting CaMKII and CaMKIV were able to immunoprecipitate gp130 phosphorylating activity from LIF-stimulated 3T3-L1 lysates. While pretreatment of cells with the MAPKK inhibitors PD98059 and U0126 blocked phosphorylation of Ser782 prior to LIF stimulation, these inhibitors did not block Ser782 phosphorylation by LIF-stimulated 3T3-L1 cell extracts in vitro. These results show that CaMKII and possibly CaMKIV phosphorylate Ser782 in the serine-based dileucine internalization motif of gp130 via a MAPK-dependent pathway.

Keywords: gp130; LIF; CaMKII; Serine-based internalization motif; Granulocyte colony stimulating factor; Phosphorylation

1. Introduction

Leukemia inhibitory factor (LIF) is a multifunctional cytokine that mediates a variety of effects in numerous cell types. For example, LIF stimulates leukemic cell differentiation and the proliferation of myeloid and platelet precursors, inhibits the differentiation of embryonic stem cells, stimulates acute phase protein synthesis in hepatocytes, inhibits lipoprotein lipase activity in adipocytes, stimulates myoblast proliferation, and converts sympathetic neurons from a noradrenergic to cholinergic phenotype ([1–3], and references therein). LIF also induces cardiac hypertrophy in rats [4,5]. LIF, ciliary neurotrophic factor, oncostatin M, interleukin-11, cardiotrophin-1, cardiotrophin-like cytokine, and interleukin-6 comprise a distinct subgroup of the cytokine superfamily that all associate with the shared signaling molecule, gp130, after initial binding to their unique low affinity α-receptor binding subunits ([6,7] and references therein).

LIF signals through a heteromeric receptor complex consisting of low affinity LIF receptor (LIFR) and gp130 [8]. LIF-stimulated dimerization of LIFR and gp130 results in activation of the Jak/Tyk family of non-receptor protein tyrosine kinases ([6], and references therein). The activated Jaks then phosphorylate and activate signal transducers and activators of transcription (STATs), which regulate a variety of cytokine-responsive genes [9–12]. Activated LIF receptors also recruit and stimulate numerous SH2-containing molecules, including phospholipase C-γ, Shc, Grb2, phosphoinositol 3-kinase, pp120,

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and the protein tyrosine phosphatase SHP-2 [13]. In cardiac cells, LIF has been shown to activate calmodulin-dependent protein kinases, CaMKII and CaMKIV due to activation of calcium channels [5], however, the mechanism for this action remains unclear.

Activated LIF receptors also stimulate the mitogen-activated protein kinase (MAPK) cascade. MAPK-mediated phosphorylation of Thr235 of NF-IL6/C-EBP-β stimulates its ability to mediate gene transcription of certain LIF-responsive genes [14]. In addition, phosphorylation of STAT1 and STAT3 by MAPK and other proline-directed Ser/Thr protein kinases can have both positive and negative effects on gene induction [6,7].

MAPK also phosphorylates human LIFR at Ser1044 [15]. This phosphorylation increases LIFR degradation and results in heterologous receptor-mediated attenuation of LIFR-stimulated gene induction due to increased receptor degradation [16]. Gp130 is also phosphorylated on serine and threonine (as well as tyrosine) residues in an agonist-dependent manner [17]. Phosphorylation of many transmembrane receptors by Ser/Thr protein kinases is known to modulate receptor activities, internalization, and downregulation in response to agonist [18].

Previously, we used mass spectrometry to identify Ser782 as the major site of agonist-stimulated serine phosphorylation of human gp130 in 3T3-L1 cells [19]. Ser782 lies adjacent to a dileucine motif which is involved in gp130 internalization [20]. Unlike LIFR, however, phosphorylation of gp130 was mediated by a protein kinase that was distinct from MAPK, but whose activation paralleled stimulation of the MAP kinase cascade in 3T3-L1 cell extracts. When compared to chimeric receptors that contained the wild-type cytoplasmic domain of gp130, chimeric gp130 receptors containing an alanine substitution at Ser782 were expressed on the cell surface at 3–8 times higher levels, and showed increased tyrosine phosphorylation in response to agonist. Furthermore, when expressed in the neuronal cell line IMR-32, the S782A mutant chimeric receptor induced VIP gene transcription at higher levels than the wild-type receptor. These results indicate that LIF stimulates phosphorylation of human gp130 at Ser782 and this site serves to regulate the cell surface expression of this receptor polypeptide. We show here that gp130 is phosphorylated on Ser782 by CaMKII and CaMKIV and we demonstrate that this process is dependent upon activation of MAPK.

2. Materials and methods

2.1. Materials

3T3-L1 cells were from the American Type Culture Collection. DMEM, fetal bovine serum, and penicillin-streptomycin were obtained from Life Technologies, Inc. (Gaithersburg, MD). Recombinant human LIF was obtained from Alomone Laboratories. Gp130 phosphoserine-specific antibody (P-S782) was obtained from Santa Cruz Biotechnology and was raised against the peptide epitope RSES^{P}TQP based on the gp130 sequence 779–785. Antibodies directed against CaMKII (H-300) and protein kinase C (PKC) μ (D-20) were also from Santa Cruz. CaMKIV antibody (#4032) was from Cell Signaling Technology. Protein G agarose was from Boehringer Mannheim (Indianapolis, IN). The CaMKII inhibitor, autocamtide-2-related-inhibitory-peptide (AIP) was from BIOMOL (Plymouth Meeting, PA). The MAPK kinase (MAPKK) inhibitors, PD98059 and U0126, were from Calbiochem (La Jolla, CA). Complete protease inhibitor was from Roche Molecular Biochemicals (Indianapolis, IN). Purified rat forebrain CaMKII was from Upstate (Lake Placid, NY). All additional supplies or materials were routinely available.

2.2. Fusion protein purification

The construction and mutagenesis of the GST–gp130 fusion protein used in this study was described previously [19]. Briefly, the cytoplasmic tail of gp130 containing amino acids 637–912 was amplified by PCR and fused to the C terminus of GST in the bacterial expression vector pGEX-3X. The induction and purification of the GST fusion protein was performed as described by Smith and Johnson [21].

2.3. Preparation of 3T3-L1 cell extract

3T3-L1 preadipocytes were cultured, stimulated, and prepared for determination of protein kinase activity as described previously [22]. Briefly, 3T3-L1 cells were grown to 80% confluency and split 1:8 on 10 cm plates and allowed to grow overnight at 37 °C in 10% CO_2, the cells were then rinsed twice with PBS and returned to the incubator in serum-free DMEM. The following day, the cells were stimulated with 100 ng/ml LIF for 10 min at 37 °C. Stimulation was quenched by rinsing the cells four times with 5 ml ice cold PBS and adding 1 ml of harvesting buffer (20 mM MOPS, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM $CaCl_2$, 1% NP-40, pH 7.2 plus Complete Protease Inhibitor). At this point, the plates were either wrapped in foil and stored at −70 °C or placed on ice to allow cell lysis. Following incubation on ice for 1 h, the cell lysates were scraped into microfuge tubes and centrifuged for 15 min at 14,000 rpm in a Beckman microfuge at 4 °C. The clarified cell extracts were then transferred to fresh tubes and either subjected to immunoprecipitation or directly assayed for protein kinase activity. In some cases, the extracts were stored in aliquots at −70 °C prior to use. Where indicated, cells were pre-incubated with 56 μM PD98059 or 8 μM

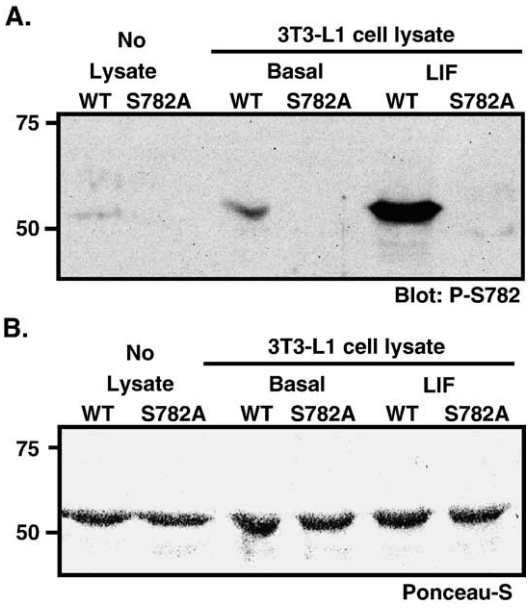

Fig. 1. Phosphorylation of Ser782 on GST–gp130 by LIF-stimulated 3T3-L1 cell extract. (A) Quiescent 3T3-L1 cells were incubated at 37 °C with diluent (phosphate-buffered saline, Basal), or LIF (100 ng/ml) for 10 min. LIF stimulated phosphorylation of GST–gp130(WT) and GST–gp130(S782A) was performed as described under Materials and methods. The kinase reactions were separated by 10% SDS-PAGE and analyzed by Western blot with P-S782 antibody. (B) Equal protein loading was verified by staining with Ponceau S. Molecular weight markers are indicated in kDa. The 54-kDa band corresponds to GST–gp130 fusion protein. The results are representative of three independent experiments repeated on separate occasions.

U0126 for 1 h prior to stimulation with 100 ng/ml LIF for 10 min.

2.4. Protein kinase assays

Phosphorylation of the cytoplasmic domain of human gp130 in clarified 3T3-L1 extracts was performed using 3 μg of fusion protein and 10 μl of cell lysate in 20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM $CaCl_2$, 0.4 μM PKI. The kinase reactions were initiated by addition of 100 μM ATP and 15 mM $MgCl_2$ and incubated overnight at 30 °C. Reactions were quenched by addition of 10 μl of 4× Laemmli sample buffer (200 mM Tris–HCl, 4% SDS, 4% β-mercaptoethanol, and 40% glycerol), boiled for 5 min, and fractionated through 10% SDS-PAGE. Phosphorylation of Ser782 was determined by Western blot using the P-S782 antibody and quantified by densitometry. Equal protein loading was verified by Ponceau-S staining.

2.5. Determination of phosphotransferase activities against gp130 fusion protein by active CaMKII

Kinase assays were performed as above using 5 and 10 ng of purified rat brain CaMKII and 8 μg/ml calmodulin in place of stimulated cell extract.

2.6. Immunoprecipitation assay

Antibodies directed against CaMKII, CaMKIV, or PKC μ were pre-incubated with protein G agarose for 30 min with rotation in 500 μl harvesting buffer and collected by touch-spin using a PicoFuge (Stratagene). Following aspiration, 1 ml of clarified cell extract was added to the immobilized antibody–protein G complex and tumbled overnight at 4 °C. Following centrifugation, supernatants were removed and assayed for gp130 phosphorylating activity. The pellets were washed twice with harvesting buffer and then twice with assay buffer and assayed for phosphorylation of GST–gp130 as described above.

3. Results

3.1. Detection of Ser782 phosphorylation using phospho-specific antibody

Using a combination of site directed mutagenesis, mass spectrometry and Edman degradation, we previously demonstrated that Ser782 is the sole site of LIF-induced serine phosphorylation on GST–gp130 fusion protein [19]. In order to more accurately and conveniently detect phosphorylation of Ser782, we used a phospho-specific antibody (P-S782) raised against a seven amino acid phospho-peptide, RSES^{-P}TQP, containing sequence corresponding to gp130 (779–785) with a phosphorylated serine at position 4, corresponding to Ser782. Fig. 1 shows the results of an in vitro kinase assay using cell extract prepared from LIF-stimulated 3T3-L1 cells and GST–gp130 as a substrate. Following phosphorylation, the kinase reactions were analyzed by Western blot using the P-S782 antibody. As shown in Fig. 1A, incubation of GST–gp130 with LIF-stimulated 3T3-L1 cell extract resulted in increased phosphorylation of GST–gp130(WT) at Ser782, while incubation with non-stimulated cell extract resulted in signal only slightly greater than the "no lysate" control. A mutant form of GST–gp130, containing an alanine substitution at Ser782, was also included in the assay. The absence of phosphorylation

Table 1
Alignment of the sequence surrounding Ser782 on gp130 with other known substrates and inhibitors of PKC μ, CaMKII, and CaMKIV

FS**R**S**E**STQPLL	gp130
PLA**R**TL**S**VAGLPGKK	syntide-2
LRR**R**RL**S**KANF	synapsin-1
KKALRR**Q**EAVDAL	AIP

Amino acid residues 777–787 of gp130 are shown with the Ser782 phosphorylation site in bold. The P−3 arginine shared by all of these sequences and the P−1 glutamic acid are also in bold. Hydrophobic residues that share sequence alignment with the P−5 phenylalanine and the P+4/P+5 dileucine internalization motif on gp130 are underlined.

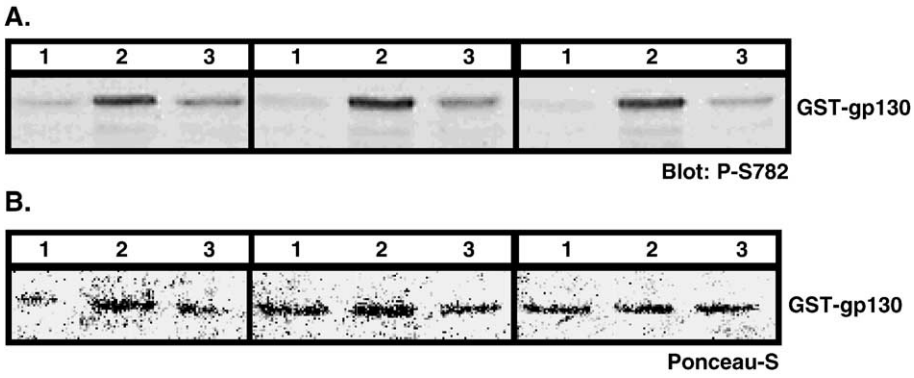

Fig. 2. Inhibition of Ser782 phosphorylation by CaMKII inhibitor, AIP. Phosphorylation of GST–gp130 fusion protein with LIF-stimulated 3T3-L1 cell extract was measured in the absence and presence of 4 μM AIP as described in Materials and methods. Lane 1, Non-stimulated cell extract; lane 2, LIF stimulated extract (100 ng/ml, 10 min at 37 °C); lane 3, LIF stimulated extract assayed in the presence of 4 μM AIP. Phosphorylation of GST–gp130 was detected by Western blot using P-S782 antibody (top). Equal protein loading was verified by staining with Ponceau S (bottom).

signal in any of the lanes containing GST–gp130(S782A) demonstrates that the P-S782 antiserum is highly specific for the phosphorylated form of GST–gp130. As shown in Fig. 1B, equal protein loading was verified by staining the blot with Ponceau S.

3.2. Phosphorylation of Ser782 is inhibited by the CaMKII inhibitor, AIP

As shown in Table 1, the Ser782 phosphorylation site on gp130 shares several similarities to syntide-2, a substrate for PKC μ [23] and CaMKII [24], synapsin-1, a known substrate for CaMKIV [24], and AIP, an inhibitor of CaMKII [25]. Like gp130, syntide-2, synapsin-1, and AIP all contain a P−3 arginine, and hydrophobic residues in the P+4 and P−5 positions. AIP also has a glutamate in the P−1 position, providing an additional feature of similarity with gp130.

Based on its sequence similarity to gp130, AIP was tested for its ability to inhibit LIF stimulated phosphorylation of Ser782 in an in vitro kinase assay with LIF-stimulated 3T3-L1 cell extract and GST–gp130 substrate. As measured by densitometry of the phosphorylated bands shown in Fig. 2, there was a 61±11% decrease in Ser782 phosphorylation when AIP was included in the kinase assay compared to LIF-stimulated phosphorylation of this site in the absence of inhibitor. The lower panel of Fig. 2 shows the Ponceau S stain of the same blot.

3.3. CaMKII phosphorylates gp130 at Ser782

Since AIP is a highly specific inhibitor for the kinase CaMKII, we tested whether purified CaMKII could phosphorylate GST–gp130 in an in vitro kinase assay. As shown in Fig. 3, purified rat forebrain CaMKII phosphorylated GST–gp130 as detected by Western blot with P-S782 antibody (upper panel). The lower panel in Fig. 3 indicates equal amounts of protein were loaded in each lane as detected by staining with Ponceau S. The inhibition of gp130 kinase activity in 3T3-L1 cell extract by AIP combined with the ability of purified CaMKII to phosphorylate GST–gp130 in vitro suggested that the kinase activity in LIF-stimulated 3T3-L1 cells is due to CaMKII. We therefore subjected LIF stimulated 3T3-L1 cell extracts to immunoprecipitation using antibodies directed against CaMKII, CaMKIV, and PKC μ and measured the ability of the immunoprecipitates to phosphorylate GST–gp130 in an in vitro kinase assay (Fig. 4). As shown in Fig. 4A, antibodies targeting CaMKII and, to some extent, CaMKIV were able to immunoprecipitate gp130 kinase activity, while the amount of activity in the PKC μ pellet was similar to the "no antibody" control. Fig. 4B shows that equal amounts of substrate was loaded in each lane as detected by Ponceau staining. In order to verify that PKC μ was actually present

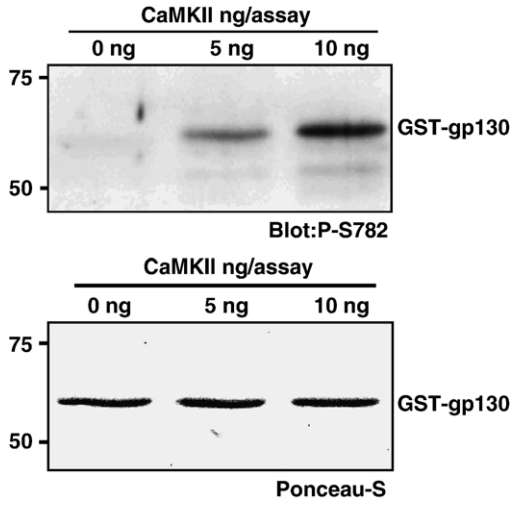

Fig. 3. Phosphorylation of GST–gp130 by purified CaMKII. GST–gp130 fusion protein was incubated with 0, 5, or 10 ng of purified rat brain CaMKII in an in vitro kinase assay as described in Materials and methods. Phosphorylation of GST–gp130 was detected by Western blot using P-S782 antibody (top) and protein loading was detected by staining with Ponceau S (bottom). Molecular weight markers (kDa) are indicated. The results are representative of three independent experiments.

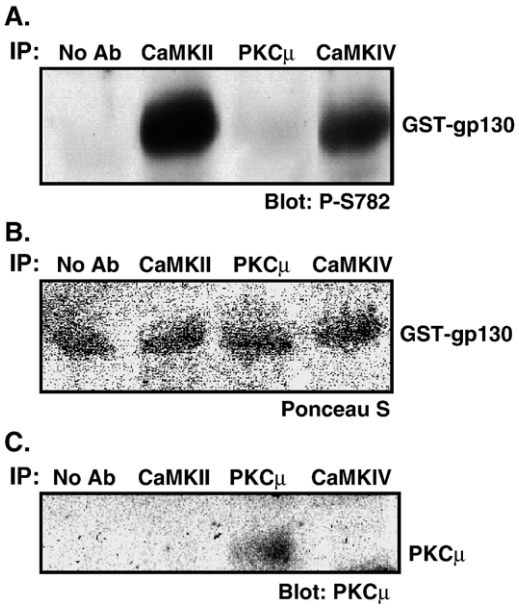

Fig. 4. Immunoprecipitation of GST–gp130 phosphorylating activity by CaMKII and CaMKIV antibodies. LIF-stimulated 3T3-L1 cell extracts were incubated with protein-G alone or with protein G and antibodies directed against CaMKII, PKC μ, or CaMKIV. The immunoprecipitates were collected and assayed for phosphorylation of GST–gp130 as described in Materials and methods. (A) Phosphorylation of GST–gp130 was detected by Western blot using P-S782 antibody. (B) Equal protein loading was verified by Ponceau S stain. (C) The same blot was stripped and re-probed with PKC μ antibody in order to confirm that PKC μ was actually present in the kinase assay performed in (A) with the PKC μ immunoprecipitate. The results are representative of four independent experiments.

in the kinase assay performed with the PKC μ immunoprecipitate, the blot was stripped and reprobed using PKC μ antiserum. As indicated in Fig. 4C, PKC μ antiserum detected a 110-kDa band in the lane containing the PKC μ immunoprecipitate but not in any of the other lanes. These results show that endogenous CaMKII and to a lesser extent CaMKIV can mediate phosphorylation of gp130 while PKC μ does not.

3.4. Treatment of 3T3-L1 cells with MAPKK inhibitor blocks phosphorylation of Ser782

Previous results have shown that while the LIFR is phosphorylated by MAPK at Ser1044, gp130 is not a MAPK substrate, as the purified MAPK ERK2 was unable to phosphorylate GST–gp130 [19]. This is consistent with the observation that the sequence surrounding Ser782 lacks a MAPK substrate consensus site [26]. Because activation of gp130 kinase activity paralleled activation of MAPK activity [19], we tested if LIF activates the gp130 kinase through a MAPK-dependent pathway. 3T3-L1 cells were treated with the MAPKK inhibitors PD98059 and U0126 prior to LIF stimulation and the resulting cell extracts were measured for gp130 phosphorylating activity in an in vitro kinase assay (Fig. 5A, right panel). As detected by Western blot with the P-S782 antibody, pre-incubation with MAPKK inhibitor resulted in a 70% decrease in LIF-stimulated gp130 phosphorylation at Ser782. In contrast, when the MAPK inhibitors were included in the in vitro kinase assay with LIF-stimulated cell extract, there was only slight inhibition of Ser782 phosphorylation (Fig. 5A, left panel). These results indicate that, while gp130 is not a substrate for MAPK, activation of the MAPK pathway is required for serine phosphorylation of gp130. Fig 5B shows the Ponceau stains of the same blots indicating equal protein loading.

4. Discussion

Ser782 on gp130 lies in a dileucine motif, which is important for internalization of gp130 [19,20]. We have previously shown that this serine is the only site of LIF-induced phosphorylation, and that mutagenesis of Ser782 results in increased gp130 cell surface expression and increased ability to mediate cytokine-dependent induction of neuronal gene expression [19]. Recently, Mitsuhashi et al.

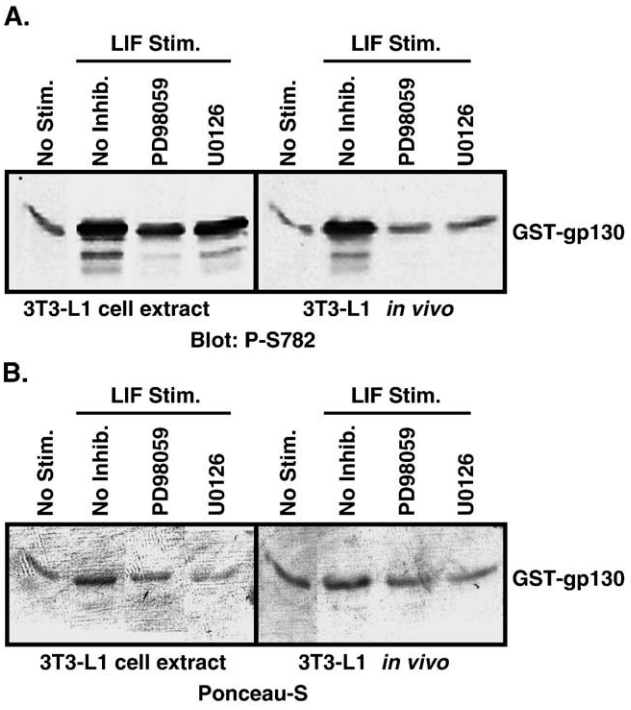

Fig. 5. Inhibition of GST–gp130 phosphorylation by the MAPKK inhibitors PD98059 and U0126. Phosphorylation of GST–gp130 was assayed as described in Materials and methods using LIF stimulated 3T3-L1 cell extract. Phosphorylation of GST–gp130 was detected by Western blot using P-S782 antibody. (A) Left panel, inhibition in cell extracts: assays were performed either in the absence of inhibitor or in the presence of PD98059 (56 μM) or U0126 (8 μM). Right panel, inhibition in intact cells: 3T3-L1 cells were pre-incubated with either PD98059 (56 μM) or U0126 (8 μM) for one h prior to stimulation with LIF. (B) The blots in (A) were stained with Ponceau S to verify equal protein loading. The results are representative of three separate experiments.

demonstrated that inhibition of the phosphatase PP2A with okadaic acid resulted in increased phosphorylation of Ser782 and increased degradation of gp130 in HepG2 cells [27]. The importance of the serine-based dileucine motif on gp130 is further supported by the demonstration that a nearly identical motif regulates GCSF receptor expression and internalization [28]. While it is not known if the serine in the GCSF receptor dileucine motif is phosphorylated, these results suggest that the serine-based motif originally identified in gp130 may be a more general internalization signal for cytokine and growth factor receptors. In this paper, we used a phosphoserine-specific antibody directed against Ser782 on gp130 to detect phosphorylation of this site in LIF-stimulated 3T3-L1 cell extract.

Phosphorylation of Ser782 could be significantly inhibited by the specific CaMKII inhibitor, AIP, raising the possibility that gp130 may be a substrate for phosphorylation by CaMKII. Consistent with this hypothesis, we found that purified CaMKII was able to phosphorylate gp130 at Ser782. Most importantly, antibodies against CaMKII, and to a lesser extent CaMKIV, were able to immunoprecipitate Ser782 kinase activity from extracts of LIF-stimulated cells, demonstrating that endogenous calmodulin-dependent kinases did indeed possess gp130 kinase activity. The specificity of this result is demonstrated by the observation that anti-PKC μ failed to immunoprecipitate gp130 phosphorylating activity, despite the fact that PKC μ and CaMKII are known to phosphorylate similar substrates. For example, they both phosphorylate syntide-2 at the same site. We have previously shown that PKC α/β also failed to phosphorylate gp130 fusion protein in vitro [19].

While gp130 is not a substrate for phosphorylation by MAPK, activation of MAPK was required for gp130 phosphorylation, as we found that two inhibitors of MAPKK could block LIF stimulation of gp130 kinase activity. In cardiac cells, LIF has been shown to activate L-type Ca^{2+} channels via phosphorylation by MAPK [29]. In addition, LIF activation of CaMKII and CaMKIV induces cardiac hypertrophy in rats [5]. In rat cardiomyocytes, LIF activation of CaMKII and CaMKIV was blocked by the calcium channel blocker, verapamil [5]. Taken together, these results suggest that in cardiac cells LIF activated MAPK mediates phosphorylation and activation of L-type calcium channels, which results in activation of CaMKII and CaMKIV. 3T3-L1 cells also contain L-type Ca^{2+} channels [30], yet surprisingly, we found that verapamil had no effect on LIF-stimulated gp130 phosphorylating activity in 3T3-L1 cells (R. M. Gibson and N. M. Nathanson, unpublished observations). This suggests that the mechanism for LIF activation of calmodulin-dependent protein kinases in 3T3-L1 cells differs from that in cardiac cells.

The inability of antibodies directed against CaMKII and CaMKIV to fully immunodeplete gp130 phosphorylating activity (R. M. Gibson and N. M. Nathanson, unpublished observations) and the inability of AIP to fully inhibit phosphorylation of gp130 in 3T3-L1 cell extract suggest that there are additional serine kinases that also phosphorylate gp130 in response to LIF stimulation. Determination of the mechanisms that activate the gp130 kinases should provide new insights into signal transduction by LIF and other members of the gp130–cytokine family.

Acknowledgements

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