Identification and Characterization of a Membrane Receptor for Proteolysis-Inducing Factor on Skeletal Muscle

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Abstract

Proteolysis-inducing factor (PIF) is a sulfated glycoprotein produced by cachexia-inducing tumors, which induces atrophy of skeletal muscle. PIF has been shown to bind specifically with high affinity ($K_d$ in nanomolar) to sarcolemma membranes from skeletal muscle of both the mouse and the pig, as well as murine myoblasts and a human muscle cell line. Ligand binding was abolished after enzymatic deglycosylation, suggesting that binding was mediated through the oligosaccharide chains. Ligand blotting of [35S]PIF to triton solubilized membranes from C2C12 cells provided evidence for a binding protein of apparent $M_r$ of $\sim 40,000$. Amino acid sequence analysis showed the PIF receptor to be a DING protein. Antisera reactive to a 19mer from the N-terminal amino acid residues of the binding protein attenuated protein degradation and activation of the ubiquitin-proteasome pathway induced by PIF in murine myotubes. In addition, the antisera was highly effective in attenuating the decrease in body weight in mice bearing the MAC16 tumor, with a significant increase in muscle wet weight due to an increase in the rate of protein synthesis, together with a reduction in protein degradation through attenuation of the increased proteasome expression and activity. These results confirm that the PIF binding protein has a functional role in muscle protein atrophy in cachexia and that it represents a potential new therapeutic target. [Cancer Res 2007;67(23):11419–27]

Introduction

Many patients with cancer suffer a severe depletion of their lean body mass, which has been linked with a reduced survival time (1). A number of cytokines, including tumor necrosis factor-$\alpha$ (TNF-$\alpha$; ref. 2), have been associated with protein depletion in cachexia, but few studies have reported a direct effect on the degradative process, although a combination of TNF-$\alpha$ with IFN-$\gamma$ has been reported to strongly reduce myosin expression in murine myotubes (3).

Unlike the cytokines, proteolysis-inducing factor (PIF) has been reported to enhance protein degradation in skeletal muscle both in vitro and in vivo (4). PIF was first detected in the urine of cancer patients with weight loss and was shown to be homologous with a material isolated from the cachexia-inducing murine tumor, MAC16 (4). Subsequent studies (5) provided evidence that tumors were the source of PIF in humans and that there was a correlation between PIF expression in tumors, its detection in urine, and weight loss. Although a recent study (6) was unable to find a correlation between the presence of PIF in urine of patients with metastatic gastric/esophageal cancer and weight loss, it is questionable whether the investigators were measuring PIF, because their Western blots showed bands at $M_r$ of 28,000 rather than 24,000 reported for PIF (4). Also, whereas PIF has been reported to comprise only 5%–10% of the total urinary protein (7), the band attributed to PIF in the mass spectrometer was a major component compared with other studies (8), wherein the band attributed to PIF was only a minor component in crude urine. PIF induces specific depletion of the lean carcass mass through an increase in protein degradation and a decrease in protein synthesis in skeletal muscle (9). Recent studies (10) show that PIF acts through the dsRNA-dependent protein kinase (PKR) to both inhibit protein synthesis and increase protein degradation in skeletal muscle.

PIF is a highly sulfated glycoprotein, consisting of a short central polypeptide chain of $M_r$ of 2,000 to 4,000, to which one O-linked sulfated oligosaccharide chain of $M_r$ at 6,000 and one N-linked sulfated oligosaccharide chain of $M_r$ at 10,000 is attached (11). The polypeptide chain is derived from the dermicidin gene (DCD), an antimicrobial peptide isolated from human sweat (12). Another peptide diffusible survival evasion peptide (or Y-P30) is a product of the same region of DCD as the PIF core peptide (13), whereas another peptide, which has been mapped to the DCD gene locus, may function as an oncogene in breast cancer (14). Unlike PIF, these peptides are not glycosylated. Both the N-linked and O-linked oligosaccharide chains are important in the biological effects of PIF (4, 11). Furthermore DCD expression does not seem sufficient to induce cachexia (15). In this study, stable forced expression of the gene for DCD resulted in secretion of a protein that was not glycosylated. However, the antibody used for detection of glycosylated PIF was raised to amino acids 44 to 62 of DCD, which would be excised if it was glycosylated to form PIF. Also, to consider that transfection with the DCD gene will necessarily lead to increased synthesis of PIF is questionable, because PIF is a highly complex sulfated glycoprotein, with 85% of the mass being complex carbohydrate, which requires the presence of both the glycosylated sugars and the relevant conjugating enzymes. Although the PIF core peptide mRNA expression is up-regulated in both tumor and adjacent normal tissue in gastroesophageal malignancy, this does not relate to prognosis or cachexia (16). This confirms that posttranslational modification of the core peptide is important to the biological effects of PIF.

Because PIF is highly negatively charged, it is unlikely to enter cells, suggesting that it must exert its effects through interaction with a membrane receptor, which in turn is linked to activation of cellular signaling pathways, such as activation of PKR (10). Because

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the sulfated oligosaccharide chains are required for biological activity, binding could be mediated through a salt linkage between the sulfate groups on PIF and basic residues on proteins in a putative receptor. The present study reports on the isolation of a membrane receptor for PIF on skeletal muscle, which has a high affinity and which seems to be linked to functional activity.

Materials and Methods

Chemicals. Fetal bovine serum (FBS) and horse serum, together with RPMI 1640 and DMEM, were purchased from Life Technologies. MAC16 monoclonal antibody was isolated from the culture medium of a hybridoma cell line (17) using a protein A Sepharose column. α-[2,6-3H]Phenylalanine (specific activity, 54 Ci mmol⁻¹), Na₂SO₄ (specific activity, 10–100 Ci mmol⁻¹), Na₂¹²⁵I (specific activity, 17.4 Ci mg⁻¹), hybod A nicotinoluse membranes, and enhanced chemiluminescence (ECL) development kits were purchased from Amersham Biosciences Ltd. All chemicals, including lectin-agarose from Triticeum vulgaris were purchased from Sigma Aldrich. Optiphase Hisafe 3 scintillation fluid was supplied by Fisons. Mouse monoclonal antibodies to 20S proteasome α-subunits and MSS1 were from Affiniti Research Products, whereas mouse monoclonal antibody to myosin heavy chain was from Novocastra. Peroxidase-conjugated goat anti-rabbit antibody and peroxidase-conjugated rabbit anti-mouse antibody were purchased from Dako Ltd.

Cell culture and tumor propagation. The C₆C₁₂ mouse myoblast cell line was grown in DMEM supplemented with 12% FBS in a humidified atmosphere of 5% CO₂ in air at 37°C. All experiments with myoblasts were performed on cells in the subconfluent state. Myotubes were formed by replacement of the growth medium of the myoblasts when they reached confluence with DMEM supplemented with 2% horse serum. Differentiation was complete within 3 to 5 days. MAC16 cells were maintained in RPMI 1640 containing 5% FBS at 37°C under an atmosphere of 5% CO₂ in air. Normal human muscle cell lines were obtained from the European Collection of Cell Cultures and were maintained in DMEM containing 2 mmol/L glutamine and 10% FBS under an atmosphere of 5% CO₂ in air. For biosynthetic labeling, the cell suspension contained Na₂SO₄ (1 μCi mL⁻¹) for 48 h in RPMI 1640 containing 1.5% dialyzed FBS (11).

Pure strain NMRI mice, bred in our own colony, were implanted in the flank with fragments of the MAC16 tumor as described (18). Weight loss was evident 10 to 12 days after tumor transplantation. Fragments of the MAC16 tumor were implanted by the same procedure. This tumor produces PIF, which has a high affinity for the PIF receptor on skeletal muscle, which has a high affinity and which seems to be linked to functional activity.

Protein synthesis and degradation in muscle. Protein synthesis was measured by the incorporation of [1-14C]phenylalanine into acid insoluble material of soleus muscle during a 2-h period as described (20). The rate of protein synthesis was calculated by dividing the amount of protein-bound radioactivity by the amount of acid-soluble radioactivity. For protein breakdown, gastrocnemius muscle was incubated in 3 mL Krebs-Hensellet buffer (pH 7.4) containing 5 mmol/L glucose and 0.5 mmol/L cytochrome c and gassed with 95% oxygen and 5% carbon dioxide. The rate of protein degradation was determined by measuring the release of tyrosine (21) over a 2-h period.

Purification of labeled PIF. Labeled PIF was purified from MAC16 cells as previously described (11) using affinity chromatography to a monoclonal antibody which recognizes the glycosylated chains of PIF (17). Further purification was achieved by hydrophobic chromatography using a Brownlee Aquapore RP-300 C₈ column and an acetonitrile in water gradient as described (4, 17). Material eluting at 55% acetonitrile was concentrated against water using an Amicon filtration cell containing a membrane filter with a molecular weight cutoff of 10,000. For [⁴⁺⁻][⁴⁺⁻]-labeled PIF, nonlabeled PIF was isolated from solid MAC16 tumors as described above and iodinated using Na₂¹²⁵I as described (4).

Membrane isolations. Sarcolemma membranes were prepared from gastrocnemius muscle of mice bearing either the MAC16 or MAC13 tumor essentially as described (22). Sarcolemma membranes from pig muscle were prepared by the same procedure and kindly donated by Dr V. Baracos (University of Alberta).

For C₆C₁₂ cells, homogenization was carried out in 20 mmol/L HEPES (pH 7.4), 1 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 1 mmol/L DTT at 4°C. The homogenate was centrifuged at 20,000 rpm for 30 min and washed with the same buffer. The pellet was used for binding studies. Adipocyte plasma membranes were prepared from adipocytes from epididymal adipose tissue of male BKW mice by a modification of the protocol of Belsham et al. (23). Essentially, plasma membranes were isolated from other components of a cell homogenate using a self-forming Percoll gradient. The membrane fractions were washed in a NaCl buffer diluted in 10 mmol/L Tris-HCl (pH 7.4), 250 mmol/L sucrose, 2 mmol/L EGTA, and 4 μmol/L PMSF at 1 to 2 mg mL⁻¹, snap frozen in liquid nitrogen and stored at −70°C until use. Hepatocyte plasma membranes were purified by a scheme similar to that for adipocytes (23), which had been modified for hepatocytes (24).

Binding studies. Membranes (200 μg protein suspended in 200 μL PBS) were incubated for 24 h at 4°C with various concentrations of [⁴⁺⁻][⁴⁺⁻]IF in 50 μL PBS as detailed in the figure legends. Bound and free radioactivity was separated by centrifugation for 5 min at 13,000 g. The binding curves were fitted to one-site and two-site hyperbolae using Graphpad PRISM v4 to obtain estimates of K_d and maximum number of binding sites (B_max). An F test was used to decide which of the models was most appropriate.

Isolation of the PIF receptor. C₆C₁₂ membranes, prepared as above, were solubilized in 1% Triton for 30 min and then dialysed against PBS overnight at 4°C. An aliquot (200 μL) of the dialysed sample was incubated with [⁴⁺⁻][⁴⁺⁻]IF for 24 h in the presence of protease inhibitors, and the PIF receptor was purified by lectin chromatography or wheat germ agglutinin (WGA) agarose, which uses the observation (17) that this lectin will bind the oligosaccharide chains of PIF. The column (1 mL bed volume; 10 mg WGA mL⁻¹) was loaded with sample and washed with 20 volumes of wash buffer [10 mmol/L Tris-HCl (pH 7.4) containing 0.02% NaN₃]. Elution of the receptor was accomplished with 10 volumes of 0.1 mol/L N-acetylglucosamine in wash buffer. Fractions (1 mL) were collected and stored at 4°C in the presence of protease inhibitors. The radioactive fractions were concentrated using a Microcon microconcentrator containing a membrane filter, with a molecular weight cutoff of M₆ at 10,000 (Amicon) and used for further analysis.

Determination of affinity constant (K_d) of PIF for monoclonal antibody. Monoclonal antibody was purified from the tissue culture supernatant of a hybridoma as described (17) using a protein A column. Serial dilutions of the antibody were made in the diluent 0.25 mol/L Tris-HCl (pH 8.5) with 2% calf serum and 1% Tween 20 to a final dilution factor of 10⁴ to 10⁶. PIF was iodinated as described (4) and diluted in the above diluent, such that 50 μL contained 2 × 10⁷ cpm. The diluted monoclonal antibody (100 μL) was dispensed into tubes followed by [⁴⁺⁻][⁴⁺⁻]IF (50 μL), and the tubes were incubated for 2 h at room temperature. Then protein A-Sepharose (100 μL) was added, and the tubes were shaken for a further 2 h. Diluent (3 mL) was added and the tubes were centrifuged, decanted, and washed with another 3 mL of diluent. The final sedimented solid phase was counted in a Gamma counter. The antibody concentration at half-maximal binding is a measure of affinity.

Competitive binding of [⁴⁺⁻][⁴⁺⁻]. C₆C₁₂ membranes (200 μg) in PBS (250 μL) were incubated with 1, 5, 10, 50, 100, 500, or 1000 ng of either monoclonal antibody, chondroitin, dermatan, or heparin sulfate and 20 mmol/L (480 ng) of [⁴⁺⁻]IF overnight at 4°C. The bound radioactivity...
was determined from the radioactivity in the pellet obtained by centrifugation at 13,000 × g for 5 min.

The \( K_{\text{aff}} \) was determined according to a modification (25) of the method of Muller (26).

\[
K_{\text{aff}} = \frac{1}{(I_t - T_i)(1 - 1.56 + 0.5b^2)}
\]

where \( I_t \) is the inhibitor concentration at 50% inhibition of PIF binding, \( T_i \) is the total PIF concentration, and \( b \) is the fraction of PIF bound in the absence of inhibitor.

**Measurement of protein breakdown in vitro.** Protein degradation in myotubes was determined by the release of \( L-[^{2,6-3H}] \)phenylalanine from cells prelabeled for 24 h as described previously (27). PIF was added at the concentrations indicated in the figure legends, and protein degradation was determined after a further 24 h in the presence of 2 nmol/L nonlabeled phenylalanine to prevent reincorporation. The rate of proteolysis was calculated by dividing the radioactivity released into the incubation medium by the protein-bound radioactivity.

**Measurement of proteasome activity.** Proteasome activity was determined fluorimetrically as the "chymotrypsin-like" enzyme activity by determining the release of aminomethyl coumarin (AMC) from the fluorogenic peptide succinyl-LLVY-AMC as described (27). Activity was measured in the absence and presence of the specific proteasome inhibitor lactacystin (10 μmol/L). Only lactacystin-suppressible activity was considered to be proteasome specific.

**Production of antiserum to the PIF receptor.** This was produced by Severn Biotech Ltd. by conjugating the 19mer peptide (5 mg) to 5 mg PPD (as carrier protein) with sulfo-SMCC through a C-terminal cysteine and subsequently immunizing two rabbits by s.c. injection of this antigen (50–200 mg) at 0.25 mL at each of four sites in Freud's adjuvant. The antibody was purified from the antisera by adding 50% saturated ammonium sulfate, followed by protein-A column chromatography (PURE1A kit, Sigma Aldrich). The antibody was purified from the antisera by adding 50% saturated ammonium sulfate, followed by protein-A column chromatography (PURE1A kit, Sigma Aldrich). The antibody titer of the purified IgG was 39.5 mg mL⁻¹ specific to the synthetic PIF receptor peptide.

**Western blotting.** Samples of cytosolic protein (5 μg) formed by centrifugation of cellular homogenates at 18,000 × g for 10 min at 4°C were resolved on 12% SDS/PAGE gels and transferred to 0.45-

**Figure 1.** Blots of binding of \([^{35}S]\)PIF to membranes isolated from C2C12 myoblasts (A), human muscle cells line Hs94MU (B), and pig sarcolemma membranes (C). D, binding of \([^{3H}]\)peptide to C2C12 membranes without treatment (■) or after incubation with PNGaseF (●) or O-glycosidase (▲) for 24 h. The experiment was repeated thrice. \( B_{\text{max}} \), nmol/mg protein.

Results

Binding studies have been conducted using \([^{35}S]\)PIF obtained by biosynthetically labeling MAC16 cells. The radioligand was purified from cell supernatants using a combination of affinity chromatography, followed by reverse phase high performance liquid chromatography on a C8 column as previously reported (3, 5). To determine whether a specific binding protein exists, ligand binding studies were performed using membranes isolated from the muscles of mice, pig, and human (Fig. 1). For murine myoblasts and the pig, the binding data best fitted a two-site hyperbola, although the second site in myoblasts was of low affinity and probably represented nonspecific binding. In all other cases, the data best fitted a one-site model. The \( K_d \) and \( B_{\text{max}} \) values presented in Fig. 1 show evidence of a high-affinity binding site (\( K_d \), 1.26 nmol/L), which was also detected on mouse skeletal muscle (\( K_d \) 1.5 ± 0.4 nmol/L) and liver (\( K_d \) 0.9 ± 0.2 nmol/L), but there was no evidence of binding in kidney or adipose tissue. There was no evidence for up-regulation of receptor number in mice bearing the MAC16 tumor with cachexia.
We have previously reported (11) that the biological activity of PIF is destroyed when the N-linked and O-linked oligosaccharide chains are removed by incubation with peptide N-glycosidase F (PNGaseF) or endo-α-N-acetylgalactosaminidase (O-glycosidase). To determine the effect of deglycosylation of PIF on binding to the receptor, experiments were conducted with peptide-labeled PIF generated by incubating MAC16 cells with L-[2, 5-3H]histidine (11). After 24-h incubation with PNGaseF or O-glycosidase binding of [3H]PIF was substantially reduced (Fig. 1D), with only nonspecific binding of the labeled polypeptide chain to the membrane.

The affinity of binding of PIF to monoclonal antibody \([K_{\text{aff}} = 10^6 \text{ (mol/L)}]^{-1}\) was found to be less than binding to either high-affinity or low-affinity sites on the muscle receptor (Fig. 2A). However, when the monoclonal antibody was added to C2C12 membranes at concentrations between 1 and 1,000 ng/250 μL, binding to the receptor was effectively inhibited \((K_d = 1.4 \times 10^{-8} \text{ mol/L; Fig. 2B})\). The monoclonal antibody was less effective at competing with membrane receptors for PIF when added after PIF \((K_d = 5.8 \times 10^{-7} \text{ mol/L; Fig. 2C})\).

Although PIF is a glycoprotein, the oligosaccharide chains have some similarity to a proteoglycan because chondroitinase avidin-biotin complex method destroys the antigenic determinants (11). This suggests that binding of PIF to the receptor may be attenuated by proteoglycans binding to the same site. To investigate this, the effect of chondroitin, dermatan, and heparan sulfate at concentrations between 5 and 5,000 ng per assay on the binding of PIF to receptors on C2C12 membranes has been determined. Of the three proteoglycans, only chondroitin sulfate showed competitive inhibition of binding (Fig. 2D) with \(K_d\) of 1.1 × 10^{-7} \text{ mol/L}.

Ligand blotting of [35S]PIF to triton solubilized membranes from C2C12 cells electrophoresed in 15% SDS/PAGE and transferred electrophoretically to nitrocellulose filters provided evidence for two binding proteins with apparent \(M_r\) of 38,000 and 40,000 (Fig. 3A). Increasing concentrations of nonlabeled PIF were capable of competitively inhibiting binding of [35S]PIF to the 38,000 protein on membranes from C2C12 cells.

**Figure 2.** A, percentage binding of [125I]PIF (20 nmol/L) at various concentrations of monoclonal antibody. The \(K_{\text{aff}}\) was calculated from half-maximal PIF binding. Competitive inhibition of PIF (20 nmol/L) binding to C2C12 membranes (20 μg) by monoclonal antibody added to membranes before PIF (B) or after PIF (C). Competitive inhibition of PIF binding to C2C12 membranes by chondroitin sulfate (D). The experiment was repeated thrice.
of displacing radioactivity from the binding proteins (Fig. 3B), confirming that binding to the receptor was specific. The analysis of this binding is shown in Fig. 1A.

The PIF-receptor complex was isolated from crude membrane preparations of murine myotubes using the lectin-binding properties of the oligosaccharide chains in PIF to T. vulgaris WGA (17). This effectively isolated the receptor predominantly as a single protein of Mr at 40,000 (Fig. 3C). Control incubations in which solubilized membranes isolated from myotubes were subjected to lectin affinity chromatography on WGA without prior incubation with PIF showed no retention of a similar protein confirming that the Mr at 40,000 material is not an endogenous glycoprotein. Separate experiments confirmed that the Mr at 40,000 material was not glycosylated, and Edman degradation analysis gave the sequence for the N-terminal amino acid residues shown below:

DINGGGATLPQKLYLIPNVL

Polyclonal antisera were generated in rabbits to a peptide derived from the first 19 amino acids of the N-terminal peptide sequence of the receptor. Using protein A-purified antisera, Western blotting detected a single band corresponding to a material or Mr of 40,000, both in WGA-purified and crude membrane preparations (Fig. 4A). The effect of addition of the antisera at concentrations between 5 and 20 μg/mL on total protein degradation induced by PIF in murine myotubes is shown in Fig. 4B. The concentration-response curve for PIF followed a bell-shaped curve, as previously reported (10, 27), with a maximum effect at 4.2 nmol/L, which is close to the binding affinity of PIF in this cell line. Partial attenuation of the effect of PIF was seen when the antisera was added at a concentration of 5 μg/mL, with complete attenuation at concentrations of 10 μg/mL or higher. Because protein degradation by PIF is mediated through the ubiquitin-proteasome pathway (28), the effect of the anti-PIF antisera on both proteasome functional activity, as determined by the chymotrypsin-like enzyme activity (Fig. 4C), as well as expression of the 20S proteasome α-subunits (Fig. 4D), was determined. As with total protein degradation preincubation with the antireceptor, antisera at concentrations of 10 μg/mL or higher completely attenuated the PIF-induced increase in chymotrypsin-like enzyme activity (Fig. 4C) and 20S proteasome α-subunits (Fig. 4D). Control experiments using a nonspecific rabbit antiserum showed that the effect was specific to the anti-PIF receptor antisera. These results confirm that antibodies to the isolated PIF receptor can attenuate the action of PIF in vitro.

To determine the effect of the antisera on the development of cachexia in vivo, mice bearing the MAC16 tumor, and with established weight loss (average, 5.4%), were treated daily with the anti-PIF receptor antibody (4 mg/kg), and the effect on body weight, tumor growth, and muscle mass was determined. Control animals received a rabbit anti-goat antibody at the same concentration. After 3 days of treatment, there was a significant reduction in the rate of weight loss of animals receiving the antireceptor antibody compared with those receiving the control antibody (Fig. 5A). There was no significant effect of the antibody on tumor growth rate (Fig. 5B). Animals receiving the antireceptor antibody showed a significant increase in the weight of soleus muscles compared with the antibody control, and this was not significantly different from weight-matched NMRI mice without tumor (Fig. 5C). Muscle mass was increased in mice treated with the antireceptor antisera due to a significant increase in protein synthesis (Fig. 5D) and attenuation of the increased protein degradation down to levels found in nontumor-bearing mice.

Figure 3. A, C2C12 membranes were solubilized in 1% Triton, and 50 μg of protein were loaded into each well of a 15% SDS/PAGE. Samples were electrophoresed and transferred electrophoretically to a nitrocellulose filter which had been preblocked overnight in blocking buffer (5% Marvel in PBS containing 0.1% Tween 20). The filters were washed thrice with PBS containing 0.1% Tween 20, air dried, reassembled, and processed for autoradiography. The 40-kDa bands were excised from each lane and counted directly for radioactivity (30). B, membrane proteins were transferred electrophoretically to a nitrocellulose filter, which was cut into individual lanes, and blotted with [35S]PIF (30 nmol/L) alone or together with 10, 20, 40, 80, 160, and 320 nmol/L unlabeled PIF in blocking buffer for 2 h at room temperature. The filter was washed thrice with PBS containing 0.1% Tween 20, air dried, reassembled, and processed for autoradiography. C, electrophoretic mobility of proteins eluting from the WGA column on 15% SDS/PAGE and stained with Coomassie blue. Lane 1, molecular weight markers; lanes 2–4, fractions eluted from the WGA column.
Functional proteasome activity, as measured by the chymotrypsin-like enzyme activity (Fig. 6B), as well as expression of MSS1, an ATPase subunit of the 19S regulator of the proteasome, were also attenuated down to the levels found in nontumor-bearing animals (Fig. 6C), whereas myosin levels were restored (Fig. 6D). These results support a role for PIF in the induction of muscle atrophy in mice bearing the MAC16 tumor, and suggest that an anti-PIF receptor antibody could be used therapeutically for the treatment of cachexia.

Discussion

For PIF to induce protein degradation in skeletal muscle, there must be a specific interaction with a receptor capable of translating the message into activation of the intracellular protein degradative system. Because PIF is a highly glycosylated and sulfated glycoprotein (1), it is likely that this will be membrane bound. The results of the present study provide evidence for specific, high-affinity binding sites for PIF in muscle cells. The affinity of binding was comparable with that found for insulin (29) and showed 10-fold to 100-fold greater affinity than binding to a monoclonal antibody, which has been used in the purification of PIF (17). However, high concentrations of the antibody were capable of displacing PIF from the membrane receptor. This would explain why high concentrations of the antibody were required to neutralize the biological effect of PIF (4, 17). As with binding of PIF to the antibody (11), binding to the receptor is probably mediated through the sulfated oligosaccharide chains, because binding was specifically inhibited by chondroitin sulfate but not by the related proteoglycans dermatan and heparan sulfate. In addition enzymatic deglycosylation resulted in the loss of specific binding of PIF to the receptor, as it has also been shown to destroy biological activity (4). The high-affinity binding probably results from electrostatic interaction between PIF and the receptor.

A plot of binding of PIF to muscle membranes from C2C12 cells and pig provided evidence for two binding sites, although in human and mouse muscle and mouse liver the data fitted to a single site model. Ligand blotting of [35S]PIF to triton solubilized membranes from C2C12 cells electrophoresed in 15% SDS/PAGE
provided evidence for two binding proteins of apparent Mr at 38,000 and 40,000. Two site models of steady-state binding have been described for a number of hormonal and nonhormonal systems. For insulin, this has been shown (30, 31) to represent negative cooperativity in the binding sites. This provides a mechanism in which binding to the receptor is favored at low concentrations of the hormone, but becomes more difficult as the concentration of the hormone is increased. Such an effect is apparent for protein degradation in C2C12 cells induced by PIF, wherein a bell-similar dose-response curve was observed. Previous studies (9) have reported similar dose-response curves for protein degradation in isolated soleus and gastrocnemius muscles induced by PIF, as well as in murine myotubes (10, 27). These results suggest negative cooperative interactions between the PIF binding sites.

We have confirmed that PIF is responsible for the loss of skeletal muscle in mice bearing the MAC16 tumor with cachexia (32). However, the number of binding sites for PIF in skeletal muscle was comparable in mice bearing the MAC16 tumor and the MAC13 tumor, which does not induce cachexia, suggesting that the induction of muscle protein degradation during the process of cachexia is not due to the up-regulation of receptors but related to the production of PIF by the tumor (4, 5), which leads to constitutive activation of muscle protein degradation.

The distribution of the PIF receptor in tissues is commensurate with a role of PIF in mediating catabolism of skeletal muscle proteins. Protein degradation rates in C2C12 myotubes were shown to increase by 50% to 90% in response to PIF, with maximal stimulation at a concentration of 4.2 nmol/L, which is close to the binding affinity of PIF to the receptor. The role of PIF receptors in the liver is probably related to its ability to increase production of IL-6, IL-8, and C-reactive protein through activation of the transcription factors nuclear factor-κB (NF-κB) and signal transducers and activators of transcription 3 (33). Induction of the ubiquitin-proteasome pathway by PIF in murine myotubes is also mediated through activation of NF-κB (27). This suggests that the PIF receptor is linked to an intracellular signaling pathway leading to activation of NF-κB in both muscle and liver.

We have used the lectin binding properties of PIF, which binds through N-acetylglucosamine residues to the lectin from T. vulgaris (WGA; ref. 17), to isolate the PIF-receptor complex. Although initial ligand binding studies suggested that there may be two proteins to which PIF binds, the isolated protein migrated as a single band on SDS/PAGE and gave an amino acid sequence analysis commensurate with it being a DING protein. DING proteins have been isolated from bacteria, plants, and animals, and have a highly conserved structure with 80% to 90% sequence identity in the N-terminal 25 to 30 amino acid residues (34). Most of these proteins have an Mr of ~40,000, as observed with the PIF receptor, and most have been identified in extracellular or cell surface locations rather than in cytoplasmic extracts. As yet, no cDNA or genomic DING sequences have been published. This has been suggested (34) to be due to a low transcript abundance, mRNA instability, rapid

Figure 5. Effect of daily administration of anti-PIF receptor IgG (4 mg kg⁻¹; n) on body weight loss (A), tumor volume (B), soleus muscle weight (C), and protein synthesis in soleus muscle (D). Control animals (●) received rabbit antigoat antibody at the same concentration. NMRI control are nontumor-bearing animals. There was no effect of the treatment on food and water intake. Differences from control animals are P < 0.05 (a), P < 0.01 (b), or P < 0.001 (c).
turnover, or by gaps in the human genome. However, it has been suggested (35) that DING proteins do not exist in eukaryotic cells and that all sequences are of _Pseudomonas_ origin due to microbial contamination. This is unlikely to be the case in the present investigation, because the murine myotubes were completely free of gross microbiological contamination and no _Pseudomonas_ could be grown either from the PIF preparations or the purified receptor. Furthermore, membrane preparations did not show the DING protein in the absence of PIF, confirming that it was isolated from the membrane after binding to PIF.

Further evidence for a role of the DING protein in a eukaryotic system comes from studies with a polyclonal antisera to the N-terminal region of the protein. Purified IgG from this antiserum completely attenuated the effect of PIF on protein degradation in gastrocnemius muscle of mice bearing the MAC16 tumor in comparison with nontumor-bearing NMRI mice (NMRI control). Western blots for the effect of the antireceptor IgG on expression of MSS1 (C) and myosin in gastrocnemius muscle (D). An actin loading control is shown underneath. Lanes 1–4, from nontumor-bearing NMRI mice (NMRI control); lanes 6–8, from MAC16 tumor-bearing mice treated with a nonspecific rabbit antibody (control); lanes 9–12, from MAC16-tumor mice treated with the antireceptor IgG. The densitometric analysis represents the average intensity of the bands determined from three separate Western blots. Differences from the antibody control are $P < 0.05$ (a), $P < 0.01$ (b), or $P < 0.001$ (c).

![Image of Figure 6](image)

_Figure 6._ Effect of the antireceptor IgG (4 mg kg$^{-1}$) or nonspecific rabbit antibody (control) on protein degradation (A) and chymotrypsin-like enzyme activity (B) in gastrocnemius muscle of mice bearing the MAC16 tumor in comparison with nontumor-bearing NMRI mice (NMRI control). Western blots for the effect of the antireceptor IgG on expression of MSS1 (C) and myosin in gastrocnemius muscle (D). An actin loading control is shown underneath. Lanes 1–4, from nontumor-bearing NMRI mice (NMRI control); lanes 6–8, from MAC16 tumor-bearing mice treated with a nonspecific rabbit antibody (control); lanes 9–12, from MAC16-tumor mice treated with the antireceptor IgG. The densitometric analysis represents the average intensity of the bands determined from three separate Western blots. Differences from the antibody control are $P < 0.05$ (a), $P < 0.01$ (b), or $P < 0.001$ (c).

Further evidence for a role of the DING protein in a eukaryotic system comes from studies with a polyclonal antisera to the N-terminal region of the protein. Purified IgG from this antiserum completely attenuated the effect of PIF on protein degradation in murine myotubes, as well as attenuating the development of cachexia in mice bearing the MAC16 tumor. Muscle mass was increased due to a significant increase in protein synthesis, whereas protein degradation was reduced down to levels found in nontumor-bearing animals. The anti-PIF IgG was effective in attenuating up-regulation of the ubiquitin-proteasome pathway induced both by PIF and the MAC16 tumor. These results suggest that the antibody binds to the PIF receptor on skeletal muscle, blocking the interaction with PIF, which would initiate the inhibition of protein synthesis and increase in protein degradation.

These results suggest that antagonists of the binding of PIF to its receptor may be useful in the treatment of muscle wasting in cancer cachexia. The binding affinity for PIF and molecular weight of the receptor seem to be similar in mouse, pig, and man, suggesting that this approach would also be appropriate for the treatment of human cachexia.

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