The inhibitor protein of phosphorylated nitrate reductase from spinach (*Spinacia oleracea*) leaves is a 14-3-3 protein

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Abstract The inhibitor protein (IP) that inactivates spinach leaf NADH:nitrate reductase (NR) has been identified for the first time as a member of the eukaryotic 14-3-3 protein family based on three lines of evidence. First, the sequence of an eight amino acid tryptic peptide, obtained from immunopurified IP, matched that of a highly conserved region of the 14-3-3 proteins. Second, an authentic member of the 14-3-3 family, recombinant *Arabidopsis* GF14ω, caused inactivation of phospho-NR in a magnesium-dependent manner identical to IP. Third, an anti-GF14 monoclonal antibody cross-reacted with IP and anti-IP monoclonal antibodies cross-reacted with GF14ω.

Key words: Nitrate reductase; Inhibitor; 14-3-3 proteins; Protein phosphorylation

1. Introduction

In higher plants, the first enzymatic step in nitrate assimilation is the reduction of nitrate to nitrite, which is catalyzed by NADH:nitrate reductase (NR; EC 1.6.6.1). It is well known that regulation of the steady-state level of NR protein is a major mechanism for the control of NR activity in leaves [1,2]. However, recent results from several laboratories have demonstrated that NR activity is also regulated by protein phosphorylation [3–7]. NR activity responds rapidly to light/dark signals and photosynthetic activity as a result of reversible regulation by protein phosphorylation (inactivation) and dephosphorylation (activation). Phospho-NR, which occurs in dark leaves, is sensitive to Mg^{2+} inhibition, whereas dephospho-NR, which occurs in illuminated leaves, is unaffected by Mg^{2+}. Spinach leaf NR is ^{32}P-labeled exclusively on seryl residues in vivo when leaves are fed [^{32}P]Pi [4]. Recently, the major regulatory phosphorylation site in spinach leaf NR was identified as Ser-543, which is located in the hinge 1 region between the cytochrome *b* domain and the molybdenum-cofactor binding domain [8,9].

Recent studies suggest that phosphorylation of NR is necessary but not sufficient to render the enzyme inactive. An inhibitor protein (IP), which apparently interacts with phospho-NR and inactivates the enzyme in the presence of Mg^{2+} or Ca^{2+}, has been partially purified from spinach leaves [10–13]. The interaction of IP with phospho-NR specifically inhibited electron flow between the heme and the molybdenum cofactor domains [9], most probably by altering the conformation of NR. Results from experiments with partially degraded NR [8] and a transgenic tobacco expressing a truncated form of NR [14] suggest that the N-terminal domain of the protein (i.e. the molybdenum cofactor domain) might be involved in the binding of IP. However, there is as yet no direct evidence that IP functions as a binding protein, and if it does, whether the phosphorylation state of NR affects binding.

Little is known about the molecular identity of IP. In our laboratory, the native IP had a molecular weight of 70 kDa on size exclusion chromatography and was suggested to be a dimer because the most highly purified preparations were enriched for proteins of ~30 kDa on SDS-PAGE [11]. The other known characteristic of IP is that it is an acidic protein with a pI of 4.4 [11].

In this paper, we unequivocally identify IP as a member of the 14-3-3 protein family, known to be directly involved in protein-protein interactions and kinase-related events in signal transduction pathways (reviewed in [15]). This provides the first evidence for a role of 14-3-3 proteins in the regulation of the enzymatic activity of a plant protein.

2. Materials and methods

2.1. Materials

Spinach (*Spinacia oleracea* cv. Bloomsdale) was grown and leaves were harvested as described [11]. All biochemicals were purchased from Sigma unless stated otherwise.

2.2. Preparation of IP as antigen

IP was extracted from 100 g of spinach leaves and purified by polyethylene glycol fractionation and sequential chromatography on DEAE-Sepharose, Blue Sepharose, and Resource Q (Pharmacia) as described [11]. The partially purified IP preparation was subjected to native gel electrophoresis in preparative nondenaturing 10% (w/v) polyacrylamide gels. IP activity was associated with the major protein band on the gel. The protein was electro-eluted (Bio-Rad) and the preparation was dialyzed into phosphate-buffered saline for use as antigen.

2.3. Production and screening on IP-specific hybridomas

About 350 μg of partially purified IP was used to immunize and boost a female BALB/c mouse with 4 injections over a period of 2 months. Serum samples were tested for their ability to immunoprecipitate IP from crude leaf extracts prior to production of the hybridomas. Production of the hybrid cell lines was performed by the Hybridoma Service Laboratory, School of Veterinary Medicine, North Carolina State University according to [16]. Approximately 700 viable hybrid cells were obtained and screened for their capacity

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Abbreviations: GF14ω, G-box binding factor of the 14-3-3 protein family; IP, inhibitor protein; NR, NADH:nitrate reductase

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to immunoprecipitate IP activity. The single cell origin of the IP-positive clones was verified by extensive subcloning using limiting dilution techniques.

2.4. Immunoprecipitation and immunoblots

Spinach leaves were extracted with 2 volumes of 50 mM MOPS-NaOH, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, 0.5 mM PMSF, and 0.1% (v/v) Triton X-100, centrifuged at 14000×*g* for 1 min and the supernatant extract filtered through 2 layers of Miracloth (Calbiochem). An aliquot (100 μl) of the extract was incubated with 20 μl of antibody cell culture supernatant for 10 min on ice. Immunocomplexes were precipitated using 5 μl of 2×Immunoprecipitin (Gibco-BRL) as the precipitating agent. After centrifugation at 14000×*g* for 1 min, the supernatant was assayed for remaining IP activity.

For immunoblot analysis, proteins were subjected to SDS-PAGE [17] and electroblotted onto Immobilon-P membrane (Millipore). The protein blots were immunostained with anti-IP cell supernatant (1:1000 dilution, unless indicated otherwise in the text) or 0.1 μl/ml mouse anti-GF14 monoclonal antibodies [18]. Immunodetection was performed with alkaline phosphatase-conjugated affinity-purified goat anti-mouse IgG and IgN antibodies followed by chemiluminescence detection according to the manufacturer's protocol (Tropix, Bedford, MA, USA).

2.5. Sequencing of a tryptic peptide derived from IP

IP was immunoprecipitated from 40 g of spinach leaves, subjected to SDS-PAGE and electroblotted onto Immobilon-P. The membrane strip containing the major isoform of IP was excised and subjected to N-terminal sequencing by automated Edman degradation using an Applied Biosystems 494A protein sequencer. Sequencing of the native protein gave no result, suggesting N-terminal blockage. To obtain internal sequence information, IP was digested with trypsin [19]. The peptide mixture was first fractionated by a microbore reverse-phase HPLC system on a Reliasil C18 column (5 μm, 300 Å, 1.0×150 mm) using a linear gradient of 5–73% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 50 μl/min. A fraction collected around 52% acetonitrile was further purified on a Vydac C18 column (5 μm, 300 Å, 1.0×150 mm) using the same HPLC system but a shallower solvent gradient (45–65% acetonitrile) to provide better separation. The fraction eluting at about 52% acetonitrile was collected and analyzed by matrix-assisted laser desorption/ionization mass spectrometry for purity and subjected to N-terminal sequencing as above.

2.6. Enzyme assays

NADH:NR activity was assayed as previously described [4]; IP activity was determined as ATP-dependent inactivation of NR [11] in the presence of Mg^{2+}. In all cases, control reactions were assayed without Mg^{2+} to ensure that total NR activity remained constant.

3. Results

3.1. Immunoprecipitation and immunoblot analysis of IP

Three independent stable hybridoma clones were isolated which produce antibodies directed against IP. However, only the clone designated 3.33 was used in the experiments presented here. Incubation of crude spinach leaf extract with culture supernatant resulted in a concentration-dependent removal of IP activity from solution in the presence of a precipitating agent (Fig. 1). The antibodies exhibited the capacity to completely precipitate IP activity, measured as ATP-dependent inactivation of NR activity, from crude leaf extracts as well as highly purified IP preparations. The monoclonal antibody was also found to completely inhibit IP activity in solution even in the absence of precipitating agent (data not shown).

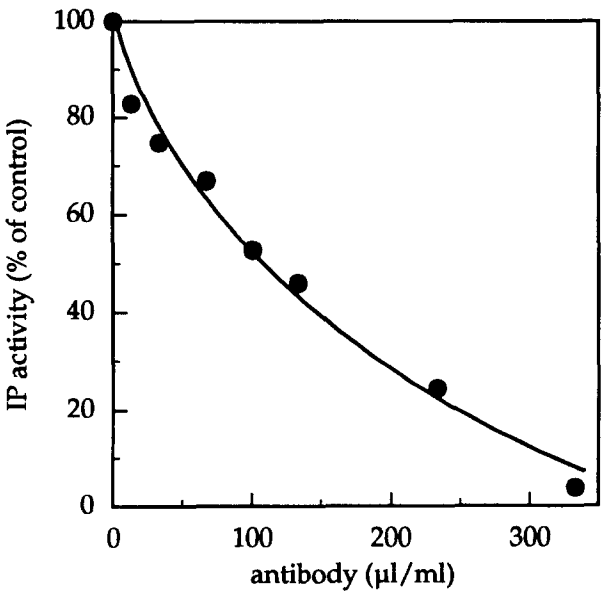

Fig. 1. Immunoprecipitation of IP from crude spinach leaf extracts with monoclonal antibody raised against IP. Immunoprecipitation was performed as described in section 2.4.

Analysis of IP immune complexes by SDS-PAGE revealed the antibody-dependent precipitation of at least three proteins from crude spinach leaf extracts (Fig. 2). The polypeptides all exhibited molecular weights in the order of 30 kDa. Inclusion of a set of protease inhibitors (Complete Protein Inhibitor Cocktail; Boehringer Mannheim, Indianapolis, IN, USA) into the extraction buffer, direct extraction of frozen leaf tissue into Laemmli sample buffer or incubation of the crude extract in the absence of protease inhibitors for 60 min at 25°C did not change the protein pattern, suggesting that proteolysis was not the cause for the multiple bands (data not shown). Transfer of the immunoprecipitates to Immobilon-P membrane and subsequent immunochemical analysis revealed that the antibody only recognized a single band at 33 kDa (Fig. 3, lane 4), corresponding to the major band in Fig. 2. Immunoblot analysis of a spinach leaf extracted directly into boiling Laemmli buffer [17] and of highly purified IP revealed the same single band at 33 kDa (Fig. 3, lanes 2 and 3, respectively). However, when the immunoblot analysis of a leaf extract was performed using the primary antibody concentration raised to a dilution of 1:20 (instead of 1:1000 as in Fig. 3), multiple bands were detected in the range of 30–37 kDa (Fig. 4, lanes 4 and 5). It is important to note that in these experiments, crude leaf extracts were electrophoresed and, thus, the immunoblots contained abundant protein over the entire molecular weight range. Clearly, nonspecific protein detection was not occurring even at the lowest dilution of monoclonal antibody. The pattern of the multiple bands detected at a low

Table 1
Recombinant GF14ω inactivates phospho-NR in the presence of Mg^{2+}

	NR activity (nmol min^{-1} ml^{-1})			
Assay:	$-Mg^{2+}$		$+Mg^{2+}$	
Preincubation:	−ATP	+ATP	−ATP	+ATP
No addition	555	550	554	545
GF14ω	547	525	562	125
IP	552	544	551	211

Partially purified NR and NR kinase, free of endogenous IP, were preincubated for 20 min with approximately 2 ng of GF14ω or IP (as indicated) in the presence or absence of 1 mM Mg·ATP. Remaining NR activity was assayed in the presence or absence of 5 mM Mg^{2+}.

antibody dilution on immunoblot analysis (Fig. 4, lanes 4 and 5) was similar to the pattern seen upon SDS-PAGE analysis of an immunoprecipitate (Fig. 2), suggesting the presence of multiple IP isoforms in leaf extracts. Apparently, the monoclonal antibody binds to an epitope that is not strictly conserved among the different isoforms. Thus, a single isoform is recognized at a very high dilution, while other isoforms are only detected at lower dilutions of antibody. Preliminary experiments showed that, when a small amount of highly purified IP was subjected to native gel electrophoresis in a 10% polyacrylamide gel, multiple protein bands could be partially separated. All bands were recognized by the anti-IP antibodies on immunoblot analysis and, when eluted from the native gel, all bands also exhibited IP activity (data not shown). Thus, IP activity is not restricted to a single protein but appears to be a property shared by several proteins with subunit molecular weights of about 30 kDa.

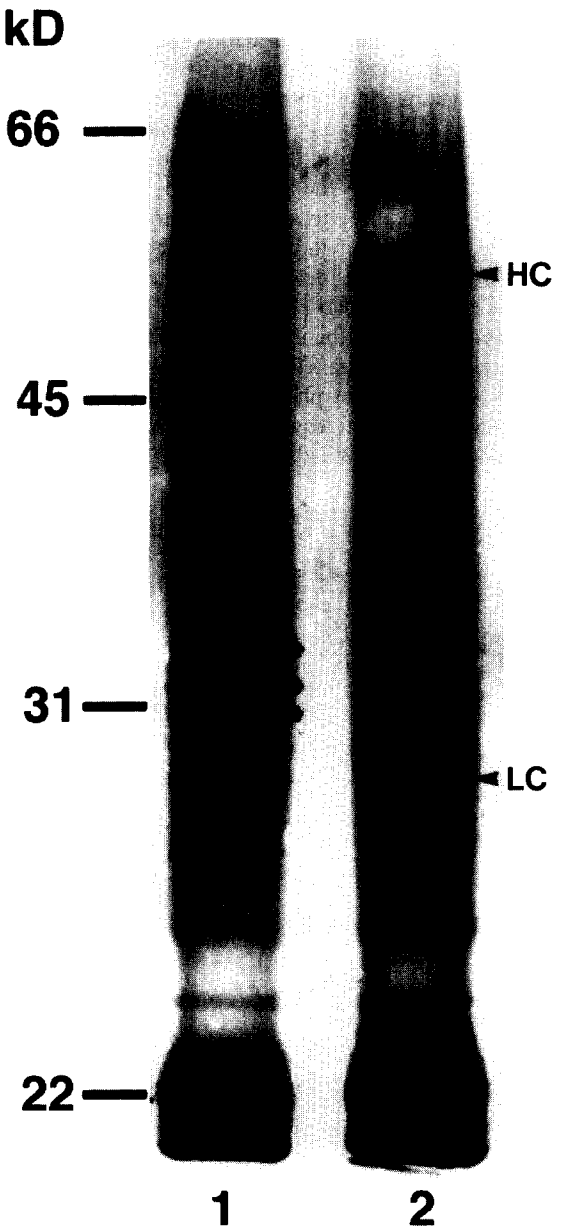

Fig. 2. SDS-PAGE analysis of IP immune complexes. Lane 1, immunoprecipitate from crude spinach leaf extracts; lane 2, immunoprecipitate from antibody cell culture supernatant. Protein bands precipitated specifically from spinach leaf extracts are marked by an asterisk, and antibody light (LC) and heavy chains (HC) are marked with arrows.

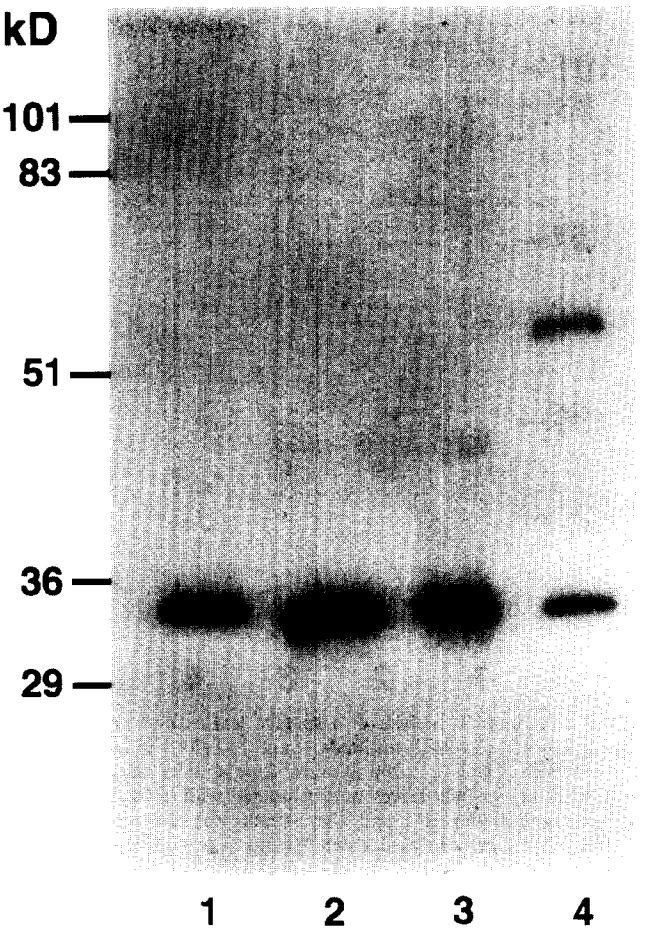

Fig. 3. Immunoblot analysis using anti-IP monoclonal antibodies. Lane 1, GF14ω (about 100 ng); lane 2, spinach leaf extract (200 μg of protein); lane 3, highly purified IP (about 100 ng); lane 4, immunoprecipitate from crude spinach leaf extract (the band at 60 kD represents the antibody heavy chain).

3.2. Sequencing of IP

Because the amino terminus of IP was found to be blocked, internal sequence information was obtained by digesting immunopurified IP with trypsin. Chromatography of the tryptic digest of IP on two different C18 columns resulted in a single peptide when analyzed by matrix-assisted laser desorption/ionization mass spectrometry. The sequence of the peptide obtained by automated Edman degradation was NLLSVAYK. A computer search for related protein sequences revealed absolute identity between the sequence derived from IP and numerous members of a protein family known as 14-3-3 proteins, cloned and sequenced from a wide range of eukaryotic organisms including mammals, insects, amphibians, yeast and plants. The amino acids correspond to residues 49–56 of 14-3-3 proteins isolated from maize, broad bean, barley, rice, pea and certain isoforms of *Arabidopsis* (accession numbers P49106, P42653, P29305, Q06967, P46266, and P42647, respectively) or to residues 42–49 of 14-3-3 proteins isolated from human, rat, bovine or sheep (accession numbers

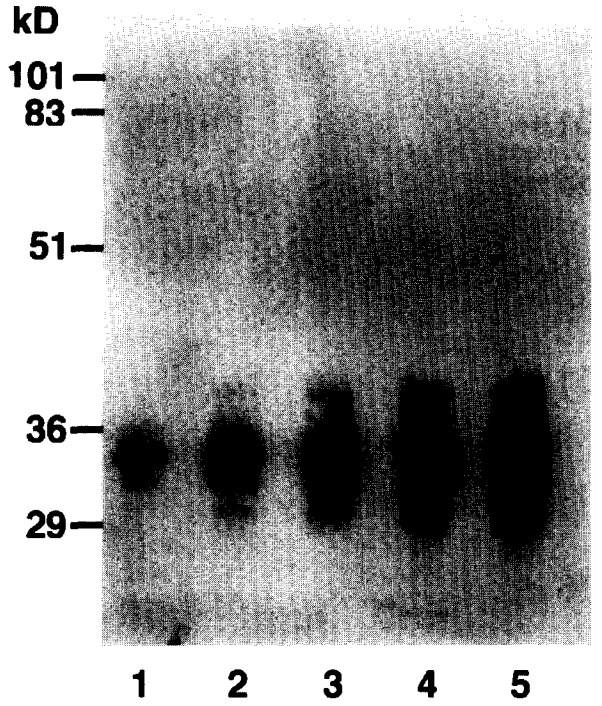

Fig. 4. Immunoblot analysis of a spinach leaf extract using different concentrations of anti-IP antibody. Lane 1, 1:2000 dilution; lane 2, 1:750 dilution; lane 3, 1:200 dilution; lane 4, 1:75 dilution; lane 5, 1:20 dilution. Spinach leaves were extracted with 4 volumes of boiling Laemmli buffer [17] and the extract was subjected to SDS-PAGE. Each lane contained about 200 μg of leaf protein.

P31947, P35216, P29359 and P29361, respectively). According to the database, the amino acid directly N-terminal to the internal sequence obtained from IP is a highly conserved arginine, indicating that the sequenced portion of IP was a full-length tryptic peptide.

3.3. *Recombinant GF14ω inactivates phospho-NR*

To verify that IP belongs to the family of 14-3-3 proteins, a recombinant 14-3-3 protein was tested for IP activity. NR and NR kinase, free of endogenous IP, were obtained by chromatography of a 5–25% polyethylene glycol fractionated leaf extract on Resource Q [11]. GF14ω, a member of the 14-3-3 family reported to be associated with a DNA-protein complex in *Arabidopsis thaliana* [20], was used in the standard assay for IP activity which is based on the ATP-dependent inactivation of spinach leaf NR. As shown in Table 1, recombinant GF14ω almost completely inactivated phospho-NR in the presence of Mg^{2+} but had no effect in the absence of Mg^{2+}, identical to the action of IP. No inactivation was seen in the absence of either IP or GF14ω. Thus, a known member of the 14-3-3 family could functionally replace IP as an inhibitor of phospho-NR in vitro.

Furthermore, immunoblotting results shown in Fig. 5 demonstrate that anti-GF14 monoclonal antibodies [18] recognized purified IP. The epitope recognized by this antibody is located within the putative EF hand calcium binding domain of GF14ω [21], indicating that IP might also contain a low-affinity calcium or divalent cation binding site. In addition, anti-IP monoclonal antibodies also recognized the GF14ω protein on an immunoblot (Fig. 3, lane 1).

4. Discussion

We have determined, for the first time, that IP is a member of the 14-3-3 family of proteins. Several lines of evidence support this postulate. First, Edman sequencing of an internal tryptic peptide derived from IP matched exactly to a strictly conserved region of the 14-3-3 proteins. Second, recombinant *Arabidopsis* GF14ω, a known member of the 14-3-3 family, could replace IP as specific inhibitor of phospho-NR (in the presence of Mg^{2+}). Third, anti-GF14 monoclonal antibodies recognized IP and anti-IP monoclonal antibodies recognized GF14ω on immunoblots. Lastly, the previously identified properties of IP [11] are consistent with those of 14-3-3 proteins; i.e. 60–70 kDa native proteins that are dimers of about 30 kDa subunit(s), with an pI of about 4.4 [22]. They were originally classified as mammalian brain proteins [23] but over the last 4 years, a number of homologues from other eukaryotic organisms have been isolated (reviewed in [15]).

While various functions for the 14-3-3 proteins emerge from the literature [15,24], many of the activities involve direct physical interaction between the 14-3-3 and the target protein. Of particular interest to the present study, a 14-3-3 protein was shown to be required to activate tryptophan and tyrosine hydroxylase upon direct binding to the phosphorylated form of the enzyme [25]. It was determined that complex formation was dependent on the phosphorylation of the target protein [26]. The action of IP in the inactivation process of NR might be of a very similar nature. IP has no effect on NR activity as long as the enzyme is not phosphorylated [9], indicating that phosphorylation may be required for the protein interaction between IP and NR. Whether phosphorylation of Ser-543

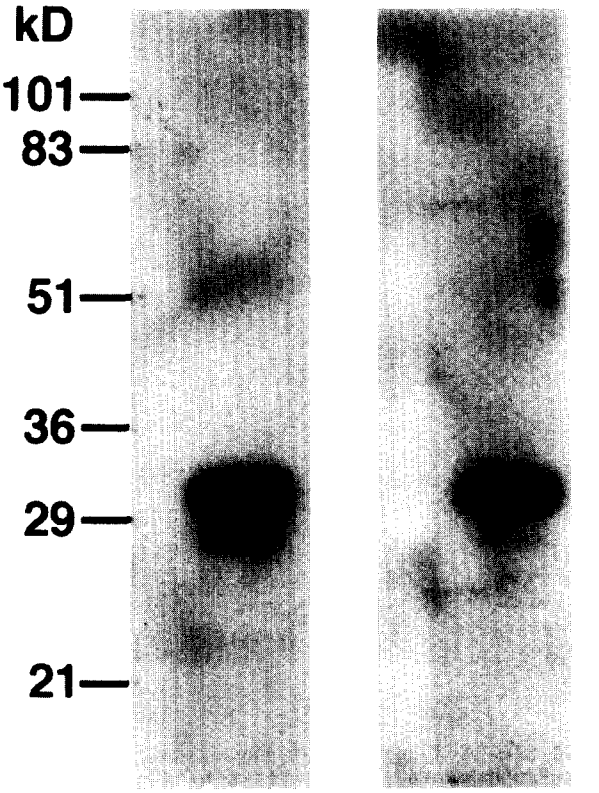

Fig. 5. Immunoblot analysis of purified IP using anti-IP monoclonal antibodies (left lane) or anti-GF14 monoclonal antibodies (right lane).

itself or a change in NR conformation caused by phosphorylation is required for the protein interaction with IP remains to be determined. Also, the role of the specific isoforms in regulating NR activity needs to be addressed in the future. However, a detailed analysis might be challenging, because 14-3-3 proteins may also form heterodimers [27] and a suitable method to separate native 14-3-3 isoforms has not yet been found. All animal and plant tissues examined to date contain several 14-3-3 isoforms. Preliminary studies in animal tissues showed that many isoforms are expressed in most tissues but tissue and organ-specific isoforms have also been isolated (reviewed in [22]).

Arabidopsis GF14ω has been demonstrated to bind calcium at a low affinity site in the C-terminal domain, resulting in an apparent change of the protein conformation [21]. The binding of Ca^{2+} was significantly reduced by Mg^{2+} [21], suggesting that the divalent cation binding site may not be specific for Ca^{2+}. The fact that anti-GF14 antibodies, which have been shown to bind to an epitope in the putative EF hand calcium-binding domain of GF14ω [21], cross-react with IP leads to the speculation that the requirement for divalent cations in NR inactivation may be due to a low affinity divalent cation binding site on IP. It can be postulated that the conformational change triggered by binding of a divalent cation is needed for effective binding of IP to phospho-NR. Thus, identification of IP as a 14-3-3 protein may explain the unusual requirement for millimolar concentrations of a divalent cation in order to inactivate phospho-NR. This, and other speculative aspects, are currently under study.

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