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In vitro Phosphorylation of the N-Terminal Half of Hordeivirus Movement Protein

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Abstract—The N-terminal half of TGB1 movement protein of poa semilatent hordeivirus, which forms a ribonucleoprotein complex involved in movement of the viral genome in the plant, and its two domains, NTD and ID, are phosphorylated *in vitro* by a fraction enriched in cell walls from *Nicotiana benthamiana*. Using a set of protein kinase inhibitors with different specificities, it was found that enzymes possessing activities of casein kinase 1, protein kinase A, and protein kinase C are involved in phosphorylation. Commercial preparations of protein kinases A and C are able to phosphorylate *in vitro* recombinant proteins corresponding to the N-terminal half of the protein and its domains NTD and ID. Phosphorylation of the NTD has no effect on the efficiency and character of its binding to RNA. However, phosphorylation of the ID leads to a decrease in its RNA-binding activity and in the ability for homological protein–protein interactions.

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Key words: hordeivirus, TGB1 movement protein, domain phosphorylation, RNA-binding activity

Protein phosphorylation is a reversible posttranslational modification that plays an important role in the regulation of many processes in eukaryotic cells. Phosphorylation influences such characteristics of proteins as enzymatic activity, stability, cell localization, and interaction with different partners. Phosphorylation/dephosphorylation of proteins also provides regulatory functions during viral infections. Modifications of both nonstructural and structural proteins of plant viruses are accompanied by changes in their functional activities, which are differently manifested at different stages of the virus life cycle, thus promoting or preventing infection of plants [1-3]. The viral genomes do not encode own protein kinases (PK). During infection, viral proteins are phosphorylated/dephosphorylated by cellular enzymes.

In the initial stage of the infection, movement proteins (MPs) of plant viruses provide for distributing the viral infection from the infected cell into non-infected neighboring cells through special intercellular membranous channels that span cell walls (CWs) – plasmodesmata (local or cell-to-cell transport). In many cases, during local transport the virus moves as a ribonucleoprotein (RNP) complex formed by the viral RNA and the MP [4-6]. The long-distance transport of viral infection through the plant vascular system, phloem, resulting in the systemic infection of the plant occurs via virions, but in some groups of viruses it can also occur via RNP complexes [4].

Phosphorylation is a frequent modification shown for MPs of many viruses from different taxonomic groups [7-12], including the MP of tomato mosaic tobamovirus [7-9], apple chlorotic leaf spot trichovirus [10], potato leaf roll polerovirus [11], cucumber mosaic cucumovirus [12], brome mosaic bromovirus [13], and potato virus X

Abbreviations: BaMV, bamboo mosaic virus; CK, casein kinase; DTT, dithiothreitol; ID, internal domain; IPTG, isopropyl-β-D-thiogalactopyranoside; MP, movement protein; NCM, nitrocellulose membrane; Ni-NTA-agarose, Ni^{2+}-nitrilotriacetate agarose; N63K, N-terminal protein half including the NTD and ID; NTD, N-terminal domain; PK, protein kinase; PSLV, poa semilatent virus; PVA, potato virus A; PVX, potato virus X; RNP, ribonucleoprotein; TGB (1-3), triple gene block encoded proteins (1-3); TMV, tobacco mosaic virus.

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(PVX) potexvirus [14]. The *in vitro* and *in vivo* modification of the tobacco mosaic tobamovirus (TMV) movement protein by serine/threonine protein kinases is best studied [1, 3, 15-25]. Phosphorylation occurs in different compartments of the cell and on different stages of the viral infection [11, 18-20, 25]. The phosphorylation of MP involves various protein kinases from plant leaves and fractions enriched with CWs or with the endoplasmic reticulum.

The identified protein kinase activities are of type casein kinase 1 (CK1) [1, 3, 20], casein kinase 2 (CK2) [8, 14, 21-23], protein kinase C (PKC) [11, 21], and some other protein kinases [9]. Enzymes with CK2 activity are involved in phosphorylation of many viral proteins including MPs of tomato mosaic virus [8], TMV [21, 22], and PVX TGB1 and coat proteins [14, 22, 23, 26]. The coat protein of potato virus A (PVA) is phosphorylated *in vivo* and *in vitro* by CK2-like enzyme [27, 28]. Phosphorylation of viral proteins or mimicked phosphorylation of a protein by a corresponding mutation is accompanied by suppression of the local movement of the viral infection [18-20, 25, 26, 29]. However, it has been shown in *in vitro* experiments that the phosphorylated form of a viral protein involved in formation of RNP complex (or virion) is characterized by a decreased efficiency of RNA binding that results in abolishment of the translational suppression of the viral RNA within such complexes [21, 23, 24, 26-29]. Despite numerous studies, the role of phosphorylation in the regulation of activities of viral proteins remains unclear in many respects.

The spread of hordeiviruses in the infected plant is provided by three MPs (TGB1, TGB2, and TGB3) encoded by a conservative module, the so-called triple movement gene block (TGB), and is realized via a non-virion RNP complex formed by TGB1 protein and viral genomic and subgenomic RNAs [30]. Non-virion RNP complexes of hordeiviruses are involved in both the cell-to-cell movement of the viral infection and the long-distance movement through the phloem. We supposed that the key role in formation of the viral RNP complex is played by the N-terminal half of the TGB1 protein consisting of two structurally and functionally different domains: the fully unstructured N-terminal domain (NTD) and the internal domain (ID) with mainly β-structure [31, 32]. The NTD of poa semilatent hordeivirus (PSLV) includes two clusters of positively charged amino acid residues responsible for the non-cooperative binding to RNA and the long-distance movement of the viral infection [33]. The ID interacts with RNA and is capable of *in vitro* self-assembly with formation of multimeric filamentous structures [31, 32]. The C-terminal half of the protein (the NTPase/helicase domain) has RNA-binding, NTPase, and RNA-helicase activities [34]. Thus, the hordeivirus TGB1 MP is a multifunctional protein performing a structural function on formation of the RNP complex and being responsible for some enzymatic activities needed for movement of the viral genome in the plant [35]. Phosphorylation of hordeivirus TGB1 protein has not been studied earlier. However, bioinformatic analysis of the N-terminal half of the protein suggests the presence of many potential sites of phosphorylation by serine/threonine protein kinases in its content.

We have shown that the N-terminal half of hordeivirus PSLV TGB1 protein and its individual domains are phosphorylated by enzymes that are present in the CW fraction from *Nicotiana benthamiana* with activities of CK1, PKA, and PKC. Phosphorylation is accompanied by inhibition of the interaction of the ID with RNA, but it does not influence the RNA-binding properties of the NTD.

MATERIALS AND METHODS

Expression of recombinant protein genes in *Escherichia coli* cells, purification of $(His)_6$-recombinant proteins by affinity chromatography on Ni-NTA-agarose, and SDS-PAGE of proteins. The previously obtained recombinant plasmids [31, 33] were used to transform strain M15 *E. coli* cells containing the high-copy repressor plasmid pRep-4. Clones expressing the recombinant protein genes corresponding to the NTD, the ID, and the N-terminal half of the TGB1 protein (N63K) were grown at 37°C overnight on standard medium 2× YT in the presence of ampicillin (100 μg/ml) and kanamycin (25 μg/ml). The overnight culture was diluted tenfold and grown at 37°C up to reaching absorption of 0.8 at 600 nm. The expression of the protein was induced by addition of IPTG (at the final concentration of 1-2 mM) for 2-4 h. The cells were separated from the culture liquid by centrifugation at 6000 rpm for 10 min in a Beckman J-21 centrifuge (Beckman, USA). The recombinant proteins fused at the N- terminus with the sequence containing six histidyl residues were subjected to chromatography on Ni-NTA-agarose according to the protocol of Qiagen (USA) under the denaturing conditions described in detail in [32]. The cells were lysed in buffer A (100 mM NaH_2PO_4 and 10 mM Tris-HCl, pH 8.0) containing 6 M guanidine hydrochloride. The supernatant after centrifugation at 6000 rpm for 10 min in the Beckman J-21 centrifuge was mixed with Ni-NTA-agarose and incubated for 1 h under gentle stirring in a column at room temperature. The column was successively washed with buffers B, C, and D (100 mM NaH_2PO_4 and 10 mM Tris-HCl) containing 8 M urea (pH 8.0, 6.3, and 5.9, respectively). The recombinant proteins were eluted from the column with buffer E (100 mM NaH_2PO_4 and 10 mM Tris-HCl) with 8 M urea (pH 4.5). The resulting fractions were analyzed by Laemmli SDS-PAGE in 15% or 20% polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue R-250.

Isolation of fraction enriched with cell walls from *N. benthamiana* plants and phosphorylation of recombinant proteins. The CW preparation was obtained as described in [16]. Leaves of *N. benthamiana* plants (without veins) were ground in liquid nitrogen into a fine powder, then homogenized in a glass homogenizer in buffer H (0.1 M Hepes, pH 7.4, 10 mM EDTA, 5 mM dithiothreitol (DTT), 1 mM PMSF) with 2% Triton X-100 (1 ml of buffer H per 1 g green mass). The suspension was centrifuged for 5 min at 1500 rpm on the Beckman J-21 centrifuge at 4°C. The pellet was washed two or three times in buffer H with 2% Triton X-100 and two times in buffer H without Triton X-100. The pellet was dissolved in buffer H without Triton X-100, and aliquots were taken, frozen in liquid nitrogen, and stored at –70°C. Aliquots thawed in ice were used only once. The standard sample contained 30 μl protein (1-1.5 μg), 5 μl of the CW preparation, 1/10 total volume of a sample of tenfold kinase buffer (200 mM Hepes, pH 7.4, 500 mM KCl, 250 mM $MgCl_2$, 1 mM DTT), and ~4 μCi [γ-^{32}P]ATP or 1.5 μl of 2 mM unlabeled ATP. Into the control sample (endogenous phosphorylation of CW proteins) the corresponding volume of H_2O was added instead of the protein solution. The mixture was incubated for 20-30 min at room temperature, and then the samples were centrifuged for 3 min at 5000 rpm. The supernatants were analyzed by Laemmli SDS-PAGE in 15% polyacrylamide gel. Samples placed into the gel were equal in protein quantity. Fractionated proteins were transferred onto a nitrocellulose membrane (NCM), which was then stained with Amido Schwarz solution and radioautographed. Inhibitors of protein kinases were added into the standard reaction mixture at the working concentration. The following inhibitors were used: KT5720; H-89 (N-[2-*p*-bromocinnamylaminoethyl]-5-isoquinoline sulfonamide); IC261 (3-[(2,4,6-trimethoxyphenyl)methylidenyl]-indolin-2-one); CKI 7 (N-(2-aminoethyl)-5-chloro-isoquinoline-8-sulfonamide); DRB (5,6-dichloro-1-β-D-ribofuranosyl benzimidazole); BIMI (bisindolylmaleimide); ML7 (1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride); H-7 (1-(5-isoquinoline sulfonyl)-2-methylpiperazine dihydrochloride); suramin; quercetin; staurosporine.

To phosphorylate the proteins with PKA from bovine heart, the minimal amount of the enzyme was dissolved in 8 μl of 40 mM DTT and maintained at room temperature for 5-10 min, then the recombinant protein (1-1.5 μg) was added in a volume not larger than 30 μl, 1/10 of the total volume of the tenfold buffer for PKA (200 mM Hepes, pH 7.6, 500 mM KCl, 100 mM $MgCl_2$) and 10 μCi [γ-^{32}P]ATP (5000 Ci/mol, 400 MBq/ml) or 1.5 μl of 2 mM unlabeled ATP. The samples were incubated for 20 min at 37°C and prepared for SDS-PAGE. Phosphorylation with PKC from rat brain was performed according to the protocol of Promega (USA): to 1 μg of the recombinant protein (in total volume of 20 μl), we added PKC, 1/5 of the total volume of fivefold activating buffer, fivefold co-activating buffer, and 10 μCi [γ-^{32}P]ATP. The sample was incubated for 30 min at 25°C and analyzed by SDS-PAGE. The ^{32}P-labeled proteins were transferred onto NCM, which was then stained with Amido Schwarz solution and radioautographed.

Determination of RNA-binding activities of proteins using electrophoretic mobility shift assay and North-Western analysis. For gel-shift assay TMV RNA (0.25 μg) was incubated on ice with increasing concentrations a protein in buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 50 mM NaCl) for 15-20 min. Samples were analyzed in 1% agarose non-denaturing gel containing ethidium bromide (1 μg/ml) in Tris-acetate buffer (40 mM Tris-CH_3COOH, pH 7.6, 1 mM EDTA). Electrophoresis was performed at 20 mA and at 40 mA after the sample entered the gel. The results were processed with the Gel-Pro program (USA). Kinetic parameters of RNA binding were calculated based on standard algorithms of the Origin 7.0 program by the formula $y = 1/(1 + K_D^n/x^n)$, where y is the amount of bound RNA, x is the protein concentration, n is the Hill coefficient, and K_D is the apparent dissociation constant. For North-Western analysis proteins fractionated in SDS-PAGE were transferred onto NCM. The NCM was incubated in buffer containing 20 mM Tris-HCl (pH 7.5), 6 M urea, and 0.05% Tween-20, then it was maintained for 1.5 h for renaturation in buffer (20 mM Tris-HCl, pH 7.5, 0.02 g/liter BSA, 0.02 g/liter Ficoll, 0.02 g/liter polyvinyl pyrrolidone), then ^{32}P-labeled TMV RNA was added, and incubation was continued for 1 h in the renaturation buffer containing 300 mM NaCl. The NCM was washed, dried, and radioautographed.

RESULTS AND DISCUSSION

Phosphorylation of N-terminal half of PSLV TGB1 protein by protein kinases from cell wall-enriched fraction from *N. benthamiana* and characterization of protein kinases. Recombinant proteins corresponding to the N-terminal half (N63K, amino acid residues 1-290 of 576 amino acid residues of the wild type TGB1 protein), NTD (amino acid residues 1-200), and ID (amino acid residues 201-290) [31] were expressed in *E. coli*, purified by affinity chromatography under denaturing conditions, renatured, and phosphorylated. The CW-enriched fractions from *N. benthamiana* were used as a source of protein kinases. As shown in Fig. 1, the CW preparation contains protein kinases capable of phosphorylating the recombinant viral proteins (Figs. 1a and 1b, lanes N63K, NTD, and ID).

To determine the type of PK, phosphorylation of the NTD and ID by the CW preparations was studied in the presence of different inhibitors of serine/threonine protein kinases. The table presents the names and concentrations of the inhibitors used, type of inhibited PK, and

data on the effect of the inhibitors on phosphorylation of the NTD and ID. Only four of 11 tested inhibitors influenced the efficiency of phosphorylation of domains of the N-terminal half of TGB1 protein.

Figure 2 presents results of the inhibition of the NTD and ID phosphorylation (the table). Of two specific inhibitors of PKA, KT5720 and H-89, only H-89 inhibited phosphorylation of the NTD and ID (Fig. 2a). ID phosphorylation was inhibited more strongly than NTD phosphorylation (Fig. 2, a, e, and f). A highly specific inhibitor of PKC, BIMI, noticeably inhibited phosphorylation of the two domains, but the inhibitory effect on the NTD was more pronounced (Fig. 2, b, e, and f). A specific inhibitor of CK1, CHI-7, inhibited NTD phosphorylation (Fig. 2c), whereas another specific inhibitor of CK1, IC261, inhibited ID phosphorylation (Fig. 2d). Both inhibitors noticeably inhibited phosphorylation of the recombinant proteins (Fig. 2, c-f), but phosphorylation of the NTD was inhibited nearly tenfold (Fig. 2e), whereas phosphorylation of the ID was inhibited virtually completely (Fig. 2f). In our experiments both inhibitors of CK2, suramin and quercetin, failed to noticeably inhibit phosphorylation (Fig. 2, e and f), although PK with CK2 activity is known to be involved in phosphorylation of many viral proteins [8, 14, 21-23, 27, 28]. Staurosporine, which is an inhibitor of a wide spectrum of serine/threonine protein kinases, including PKC, also displayed no inhibitory effect. This result is in agreement with data on the resistance to staurosporine of protein kinase activity associated with CWs [22].

The intensity of domain phosphorylation normalized per mol protein was calculated from the data presented in Figs. 1 and 2, and it was found that the ID is phosphorylated two to five times more effectively than the NTD. Figure 2a shows that not only the ID monomer is phosphorylated, but also the ID dimer and oligomer are phosphorylated to a small extent. We showed earlier that the ID is mainly present as oligomeric/multimeric structures and that the protein dimers are retained upon heating with SDS and during SDS-PAGE [31, 32]. The ID is clearly phosphorylated inside these structures, and this suggests that the phosphorylation site/sites are exposed on their surface. In preparations of phosphorylated ID, dimers are absent or their content is noticeably decreased (Fig. 1). We think that phosphorylation decreases the resistance of dimers/oligomers to treatment with SDS, which suggests the weakening of protein–protein interactions.

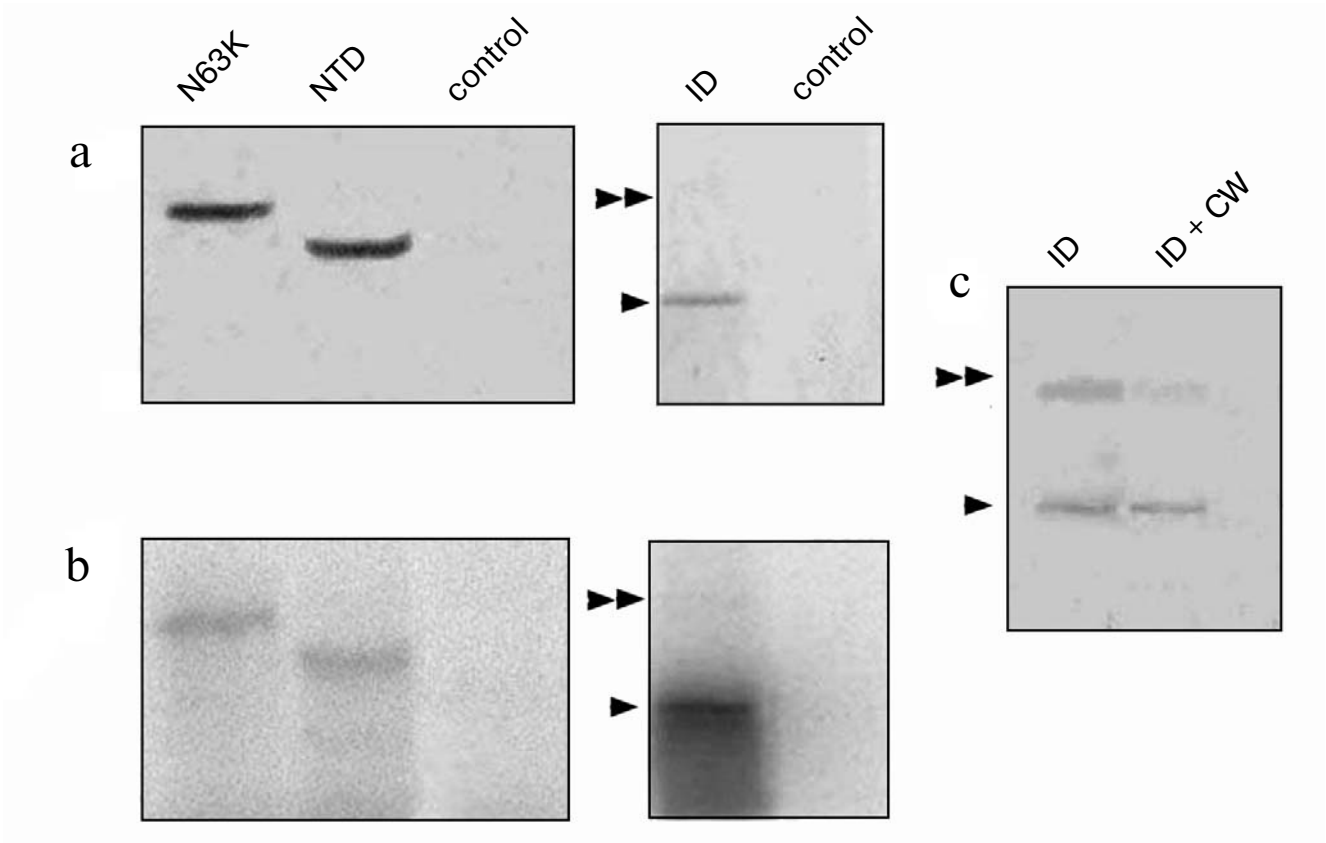

Fig. 1. Phosphorylation of the N-terminal half of the PSLV TGB1 movement protein (N63K) and its domains NTD and ID by the CW-enriched fraction from *N. benthamiana*. a) NCM stained with Amido Schwarz; b) radioautograph. Control, the sample without recombinant proteins (endogenous phosphorylation of CW proteins). c) Equal amounts of unlabeled non-phosphorylated and phosphorylated ID were transferred onto the NCM after SDS-PAGE and stained with Amido Schwarz. The arrows indicate positions of the ID monomer and dimer.

Influence of specific inhibitors of serine/threonine protein kinases on phosphorylation of domains in the N-terminal half of PSLV TGB1 protein

Inhibitor	Protein kinase type	Concentration in sample	NTD	ID
KT5729	PKA	10 μM	–	–
H89	PKA	100 μM	+	+
IC261	CK1	1 mM	–	+
CKI7	CK1	1 mM	+	–
DRB	CK2	1 mM	–	–
Suramin	CK2	20 μM, 100 μM	–	–
Quercetin	CK2	1 mM	–	–
BIMI	PKC	100 μM	+	+
ML7	MLCK	100 μM	–	–
Staurosporine	serine/threonine PK	1 μM, 10 μM	–	–
H7	cerine/threonine PK	2 mM	–	–

Note: MLCK, myosin light chain kinase; +, inhibition of phosphorylation; –, no inhibition detected.

Based on our findings, we have come to some conclusions. First, phosphorylation of the N-terminal half of PSLV TGB1 protein by the fraction enriched with CWs from *N. benthamiana* occurs more than one PK. Second, PK activities from the CW fraction of *N. benthamiana* that are able *in vitro* to phosphorylate the domains of the N-terminal half of PSLV TGB1 protein belong to the CK1, PKA, and PKC type.

It is especially interesting that PK with CK1 activity is involved in phosphorylation of the TGB1 movement protein domains. Earlier, it was shown that PK (PAPK1) associated with CWs from *N. tabacum* and *Arabidopsis thaliana* and belonging to the highly conservative CKI family of eukaryotes (including plants) phosphorylates the TMV MP and some endogenous plant non-cell-autonomous proteins that are able, similarly to MPs of plant viruses, move from cell to cell [3, 20]. It is important that PAPK1 co-localizes with the TMV MP in CW plasmodesmata and as supposed fulfils a regulatory function in the movement of macromolecules between the plant cells [20].

The presence in plants of protein kinases with biochemical features of PKC was shown earlier [36, 37]. Data on specific suppression of the polerovirus MP phosphorylation by membrane preparations from tobacco leaves [11] in the presence of a PKC peptide inhibitor allowed to use mammalian PKC for *in vitro* phosphorylation of plant virus proteins [21, 24, 26]. However, despite the wide distribution and important role of PKA and PKC in animal and yeast cells, such protein kinases have not yet been found in plants, although specific phosphorylation sites for these protein kinases have been found in many plant proteins [38, 39].

Using the GPS 2.0 computer program (http://gps.biocuckoo.org/), potential phosphorylation sites of CK1, PKA, and PKC were also revealed in the NTD and ID of TGB1 protein (Fig. 3). Phosphorylation of N63K, NTD, and ID by commercial preparations of PKA and PKC *in vitro* confirms the involvement of plant enzymes with similar activities in the phosphorylation of viral proteins (Fig. 4). In the sequence of the N-terminal half of PSLV TGB1 protein, potential sites for CK2 phosphorylation are also predicted (at residues T27, T134, and S191). However, as it was already mentioned we failed to obtain data on involvement of this PK in phosphorylation of N63K by the CW-enriched fraction. This might be explained by the preferential localization of CK2 in the cytosol [8, 14, 26-28], although CK2 has been also found in plant CWs [21-23].

Influence of phosphorylation on RNA-binding activity of the domains. The RNA-binding activity is an important feature of a protein involved in formation of a movement non-virion RNP. We earlier showed that both domains nonspecifically bind RNA, but the ID binds with RNA cooperatively, and the NTD binds with RNA non-cooperatively [31]. The influence of phosphorylation on the RNA-binding activity of the domains was studied by gel-shift assay and by North-Western analysis. For gel-shift assay the domains were phosphorylated using the CW fraction, whereas for North-Western analysis the protein was also phosphorylated by PKA. Phosphorylation did not influence the type (non-cooperative) and efficiency of RNA binding by the NTD (data not shown; Fig. 5a). On the contrary, the phosphorylated ID bound RNA with lower efficiency (Fig. 5, b-d). The RNA-binding activities of the phosphorylated and non-phosphorylated ID

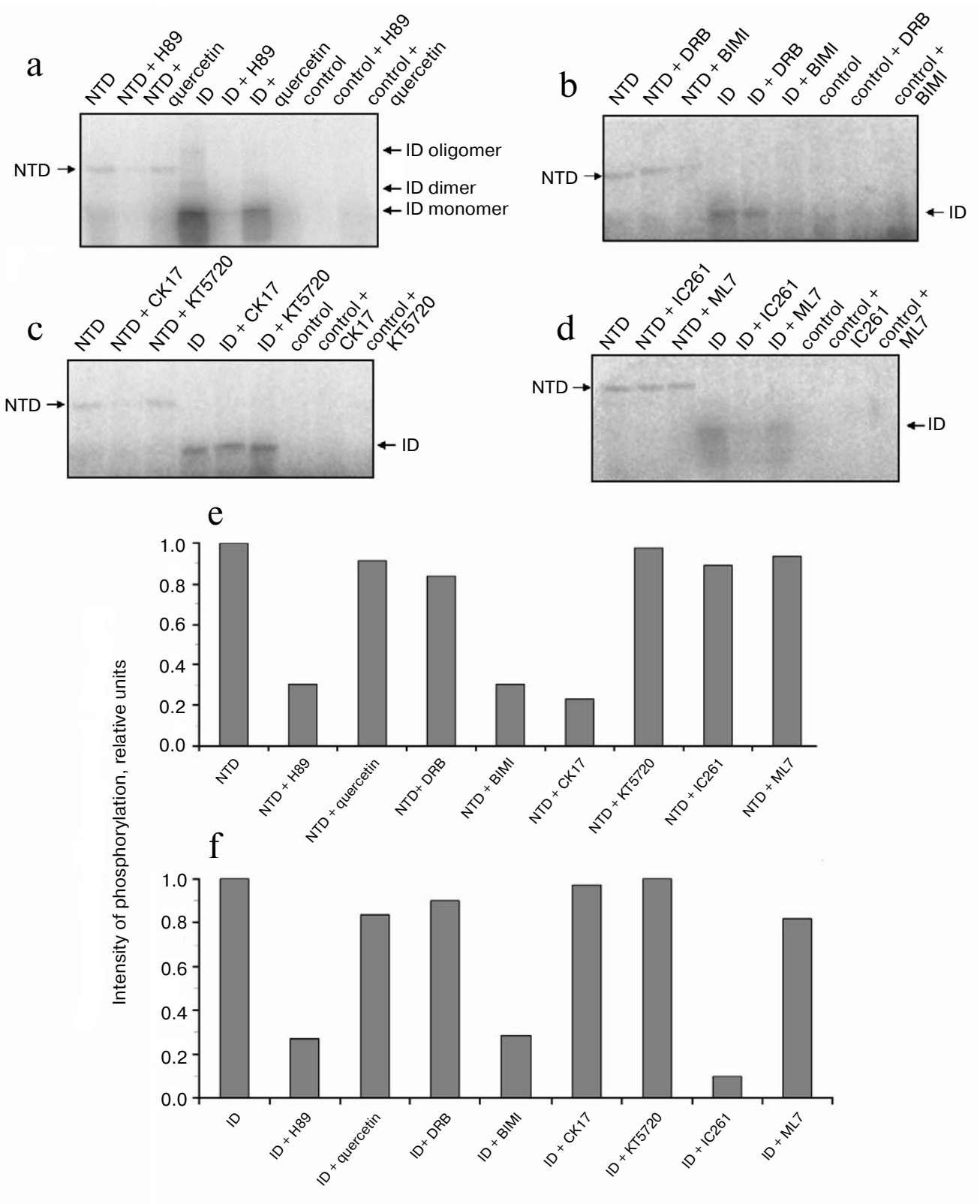

Fig. 2. Phosphorylation of the NTD and ID in the presence of specific inhibitors of protein kinases: a) H89 (100 μM) and quercetin (1 mM); b) DRB (1 mM) and BIMI (100 μM); c) CK17 (1 mM) and KT5720 (10 μM); d) IC261 (1 mM) and ML7 (100 μM). Control, sample without recombinant protein in the presence of the corresponding inhibitor. e, f) Histograms illustrating effects of different inhibitors on phosphorylation of the NTD and ID. The intensity of protein phosphorylation in the absence of inhibitors is taken as unity.

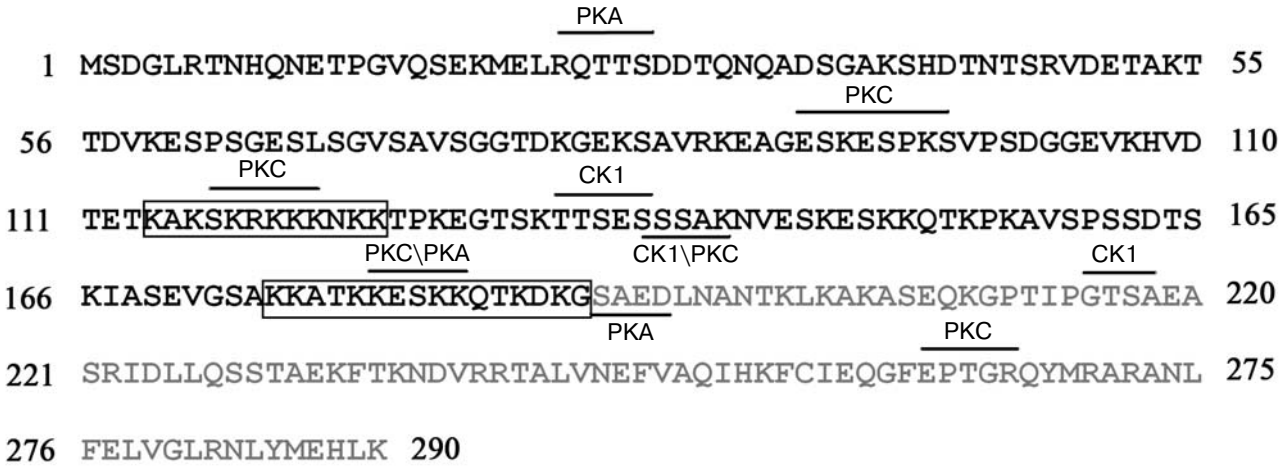

Fig. 3. Prediction of potential phosphorylation sites in the N-terminal half of the PSLV TGB1 protein. Bioinformatic analysis of the amino acid sequence using the GPS 2.0 program (http://gps.biocuckoo.org/). The NTD sequence is shown in black, the ID sequence is shown in gray. Clusters of positively charged amino acids A and B are outlined with rectangles. Potential phosphorylation sites are indicated with lines.

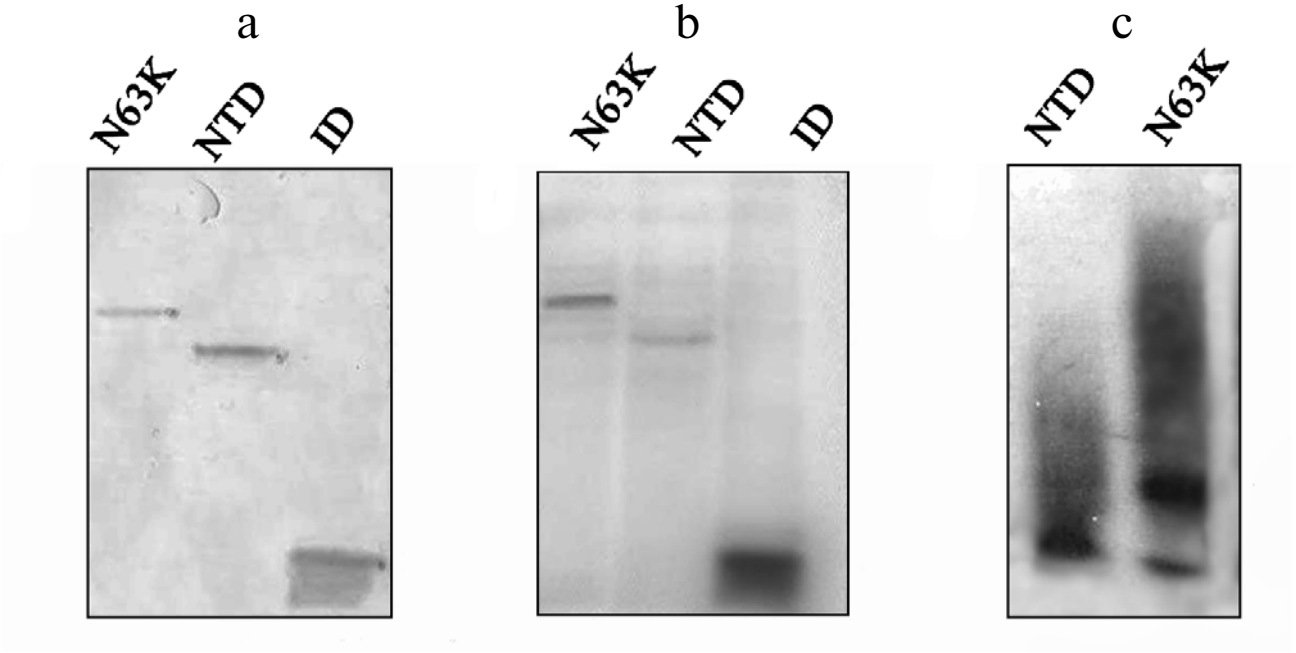

Fig. 4. Phosphorylation of N63K, NTD, and ID by commercial preparations of PKA (a, b) and PKC (c). The ^{32}P-labeled recombinant proteins were fractionated by SDS-PAGE in 15% (a, b) or in 10% (c) gels and transferred onto NCM, which was then stained with Amido Schwarz (a) and radioautographed (b, c).

were nearly fivefold different when analyzed by North-Western method (Fig. 5b) and 2.0-2.5-fold when analyzed by gel-shift assay (Fig. 5c) (K_D = 1.3 ± 0.25 and 3.0 ± 0.40 μM, respectively). However, RNA is completely bound by the non-phosphorylated ID at the protein/RNA molar ratio 200 : 1 (Fig. 5c), whereas the phosphorylated protein did not completely bind to RNA at the studied ratios (Fig. 5d).

We did not reveal significant changes in the values of *n*, an indicator of cooperativity of protein binding with RNA: $n = 1.7 \pm 0.30$ and 1.6 ± 0.35 for non-phosphorylated and phosphorylated proteins, respectively. This indicates the cooperative character of the interaction. However, the absence or decrease of the dimer content in the preparations of phosphorylated ID (Fig. 1) suggests the inhibition of interactions between phosphorylated ID molecules. This result correlates with data obtained for phosphorylated RNA-binding protein encoded by the satellite RNA of bamboo mosaic potexvirus (BaMV) [29].

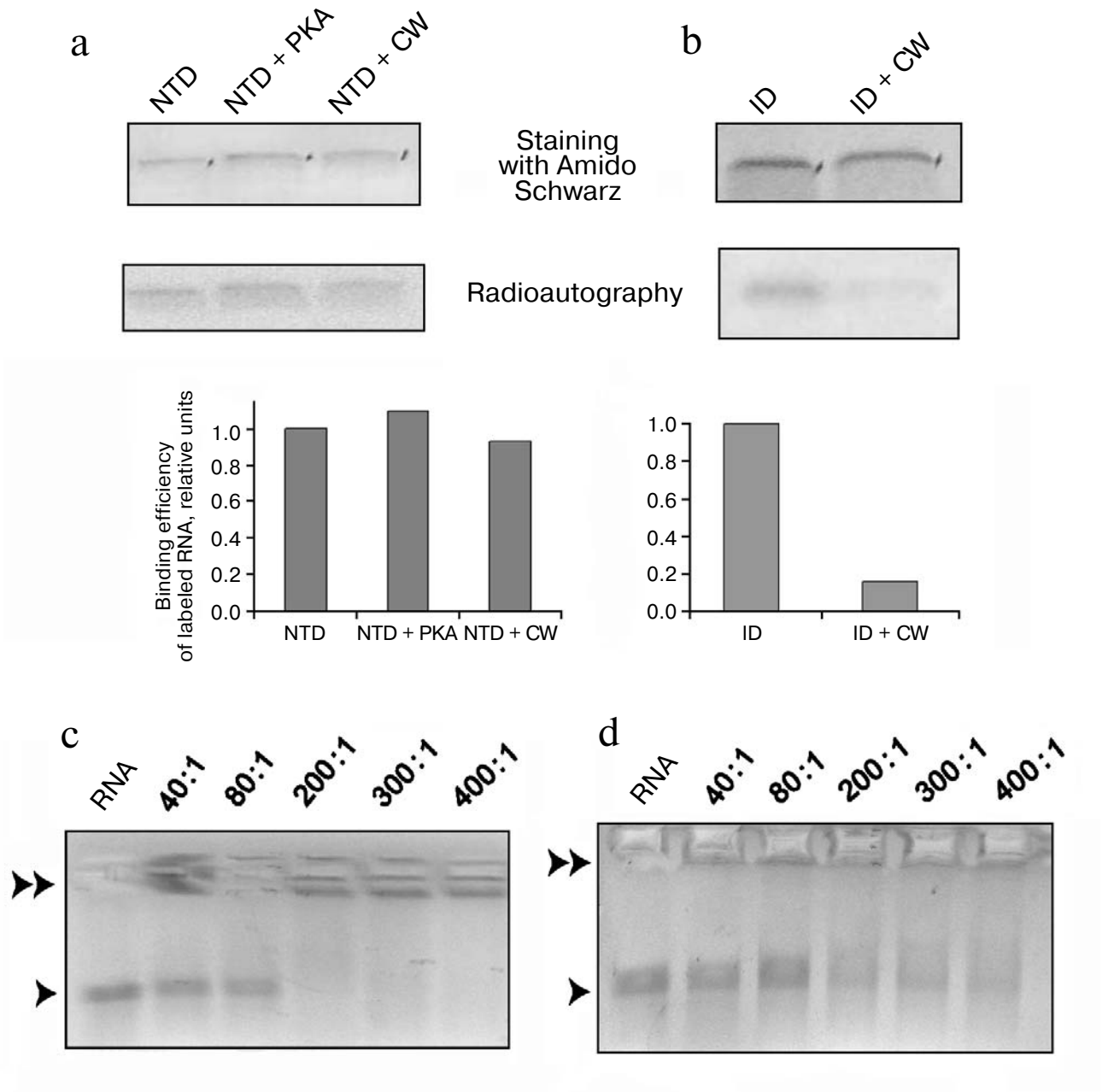

Fig. 5. RNA-binding activities of non-phosphorylated and phosphorylated forms of the NTD and ID. North-Western analysis of the NTD (a) and ID (b). Equal amounts of non-phosphorylated and phosphorylated recombinant proteins were fractionated by SDS-PAGE, transferred onto NCM, and incubated with ^{32}P-labeled TMV RNA in the presence of 300 mM NaCl. NCM stained with Amido Schwarz (upper panel), radioautograph (middle panel), and histogram of calculation of data on RNA binding (lower panel). In the histogram along the ordinate axis the efficiency of ^{32}P-RNA binding is presented in relative units. The RNA binding by the non-phosphorylated domain is taken as unity. Gel-shift assay of non-phosphorylated (c) and phosphorylated ID (d). Increasing protein amounts were incubated with the same RNA amount, and the resulting complexes were fractionated in 1% agarose gel in the presence of ethidium bromide. The results were viewed under UV. The protein/RNA molar ratios are shown for each lane. The locations of free RNA and RNA within complexes are shown by one and two arrows, respectively.

The decrease in efficiency of RNA binding on phosphorylation of proteins involved in the movement of the viral genomes has been shown for many viral proteins, including MP of TMV [21], PVX coat protein [23, 26], PVA coat protein [27, 28], and the nonstructural protein P20 producing an RNP complex with satellite RNA of BaMV [29]. The binding efficiency decreased 2-3-fold for proteins phosphorylated in the presence of CWs [21], whereas for recombinant proteins with a substitution imitating the phosphorylated state this effect was more significant (tenfold and higher) [29]. Nevertheless, in all these experiments the phosphorylation of proteins was

accompanied by translational activation of RNA within RNP complexes. This effect was demonstrated for the TMV MP phosphorylated by PKC or protein kinases associated with CWs [21], for the P20 protein encoded by the satellite RNA of BaMV (an imitation of the protein phosphorylated state [29]), for the PVX coat protein phosphorylated in the content of virion by PKC, a mixture of CK1 and CK2, or by cytoplasmic protein kinases [24, 26], and for the PVA coat protein phosphorylated by CK2 [27, 28].

Because the PSLV TGB1 protein forms the viral RNP complex for the movement of the viral genome from cell to cell, it seems very probable that its phosphorylation by the CW-associated PK is also necessary for the translational activation of viral RNA. The internal domain ID can form elongated filamentous multimeric particles, which seem to be a structural basis for the RNP complex organization [31, 32]. The domain interaction with RNA initiates its self-assembly into these filamentous structures [32]. According to our preliminary data, phosphorylation leads to changes in the secondary structure and conformation of the ID, which might disturb the interaction with RNA and influence the efficiency of protein–protein interactions within the full-size TGB1 protein. These changes could promote destabilization of the RNP complex during translocation through the CW plasmodesmata and be responsible for transition of the viral RNA into the neighboring cell in the translatable form, as supposed earlier for the RNP complex formed by the TMV MP and RNA [21].

Thus, we have shown for the first time that protein kinases associated with CWs of *N. benthamiana*, which possess activities similar to those of CK1, PKA, and PKC, are able to phosphorylate the N-terminal half of PSLV TGB1 protein that results in suppression of the RNA-binding activity and of the ability for homologous interactions of its domain ID participating in multimerization/self-assembly of PSLV TGB1 protein.

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