Identification of Plectin as a Substrate of p34<sup>Cdc2</sup> Kinase and Mapping of a Single Phosphorylation Site

(Received for publication, November 15, 1995, and in revised form, January 18, 1996)

Nicole Malecz, Roland Foisner, Christine Stadler, and Gerhard Wiche
From the Institute of Biochemistry and Molecular Cell Biology, University of Vienna, Biocenter, Dr. Bohrgasse 9, A-1030 Vienna, Austria

Plectin is an abundant cytoskeletal protein of exceptionally large size. Electron microscopy of purified plectin molecules (1) and structure prediction based on the domains and sequencing of rat plectin cDNA (2) revealed an extended central rod and two flanking globular domains as distinctive structural features. Its subcellular distribution, in particular its partial co-localization with intermediate filaments (3,4) and prominent occurrence at plasma membrane attachment sites of IFs and microfilaments, and the identification of numerous specific binding partners at the molecular level (reviewed in Refs. 3 and 4) suggested that plectin might be involved in versatile cytoplasmic cross-linking functions. In a first approach to characterize plectin’s various binding domains, transient transfection experiments indicated a role of the C-terminal globular domain in the binding to vimentin (5).

As a prominent phosphoprotein plectin was found to be an in vivo target of a Ca<sup>2+</sup>-calmodulin-dependent kinase and of protein kinases A and C (6-8). In vitro studies demonstrated that plectin’s capacity to bind to IF proteins, such as vimentin and lamin B, were differentially influenced by phosphorylation (8), suggesting that distinct protein kinases were involved in regulating at least some of plectin’s interactions.

In view of plectin’s proposed role as a cytoplasmic cross-linking protein, a specific regulation of its binding activities would seem of particular importance during mitosis, when drastic structural rearrangements of the cytoskeleton, including IF networks, take place. In fact, two of plectin’s well characterized binding partners, vimentin and lamin B, have been shown to act as direct targets of mitotic cyclin-dependent p34<sup>Cdc2</sup> kinase. Phosphorylation of vimentin subunits by p34<sup>Cdc2</sup> kinase at the onset of mitosis has been shown to correlate with the disassembly of the vimentin network (9,10), and the phosphorylation of lamin B by p34<sup>Cdc2</sup> is directly related to the disassembly of the nuclear lamina occurring at the same time, as demonstrated in vivo (11,12) and in vitro (13,14). We report here that plectin, too, serves as a specific substrate of p34<sup>Cdc2</sup> kinase, and we show that a single threonine residue residing in the C-terminal globular domain serves as a target site.

MATERIALS AND METHODS

Cell Culture, Synchronization and Metabolic Labeling—Chinese hamster ovary cells (clone CHO K1) were grown to a density of about 80% in plastic culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS (Life Technologies, Inc., Paisley, United Kingdom) and 50 μg/ml penicillin and streptomycin (Life Technologies, Inc.). For synchronization, cells were arrested at G<sub>0</sub>S by an overnight incubation in the presence of 2 μM thymidine (15) in Joklik’s modified minimum essential medium (S-MEM, Life Technologies, Inc.) supplemented with 10% FCS, a nonessential amino acid mix (1:100, Life Technologies, Inc.), 50 μM Hepes/NaOH, pH 7.4, and 50 μg/ml penicillin and streptomycin. To obtain mitotic cells, they were released from the thymidine block for 3 h in complete S-MEM and allowed to grow for another 4 h in S-MEM containing 0.2 μg/ml nocodazole. For cells enriched in S phase, the cultures were released from the thymidine block in complete S-MEM for 4 h. Cells were washed with phosphate-free DMEM (Sigma, Deisenhofen, Germany) and metabolically labeled with 0.2 μCi/ml [3<sup>2</sup>P]orthophosphate (carrier-free, DuPont NEN, Dreieich, Germany) in phosphate-free DMEM supplemented with 25 μM Hepes/NaOH, pH 7.4, and 10% FCS dialyzed against 25 μM Hepes/NaOH, pH 7.4 for 2 h. Nocodazole-arrested mitotic cells were harvested by mechanical agitation; cells still attached after nocodazole treatment (mainly in G<sub>2</sub> phase, as well as cells in S phase were scraped off. Interphase cell pellets from one 75-cm<sup>2</sup> Petri dish or mitotic cell pellets collected from three Petri dishes were washed in phosphate-buffered saline and dissolved in 300 μl of buffer A (50 μM Hepes/NaOH, pH 7.0, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.5% Triton X-100, 100 mM NaCl, 0.1 mM dithiothreitol) supplemented with (i) protease inhibitors: PMSF (1 mM), benzamidine (10 mM), aprotonin (10 μg/ml), pepstatin (10 μg/ml), and leupeptin (10 μg/ml); (ii) phosphatase inhibitors: microcystin (10 μM), okadaic acid (1 μM), calyculin (100 μM) (all from Life Technologies, Inc.), NaF (10 mM), sodium pyrophosphate (10 mM), β-glycerophosphate (5 mM), dithiothreitol (1 mM), and PMSF (1 mM).
For the preparation of p34cdc2 with 100 μM ATP and protease inhibitor mixture (kindly provided by L. Gerace). The immunocomplex was precipitated by a 2-h incubation with 200 μl of protein A-Sepharose, washed in RIPA buffer plus 0.1% SDS, and dissolved in 10 volumes of electrophoresis sample buffer.

Preparation of Protein Kinases—Nocodazole-arrested CHO cells (grown in 400-cm² roller bottles) or S phase cells (grown in one roller bottle) were collected and lysed in 1 ml of buffer A (containing protease inhibitor mixture) by 3×10-s Ultra-turrax treatment at maximum speed. The lysates were incubated with 20 μg/ml DNase and 20 μg/ml RNase for 10 min, and centrifuged for 20 min at 35,000 rpm in a Beckman 65 rotor (Beckman Instruments Inc., Palo Alto, CA). Mitotic and interphase cell lysates were diluted to the same protein concentration, mixed with 16% glycerol, and frozen in liquid nitrogen. Cdk type kinase isoforms were isolated from the cell lysates supernatants by incubation with 100 μl of p13-Sepharose beads (18) overnight at 4°C. The beads were sedimented, washed in kinase buffer (20 mM Heps/NaOH, pH 7.0, 10 mM MgCl₂) and frozen in the same buffer, containing 16% glycerol. For the preparation of the p34cdc2 kinase, 1 ml of mitotic cell lysate was mixed with 100 μl of RIPA buffer plus 0.1% SDS, and incubated overnight at 4°C with 20 μl of a rabbit antiserum generated against a synthetic peptide representing the C terminus of human p34cdc2 kinase (kindly provided by L. Gerace). The immunocomplex was precipitated by adding 200 μl of 30% protein A-Sepharose washed in kinase buffer, and frozen in this buffer plus 16% glycerol.

cDNA Constructs—To obtain cDNA inserts for the plasmids pNM1, pNM2, pNM4, pMN5, pTH4, pTFV5, and pTH6, PCR under standard conditions (19) was performed using plasmid pAD14 (5) as template. Primers were constructed in such a way that the S primer carried an additional EcoRI site, and the 3' primer an additional Xbal site, to facilitate site-directed cloning. PCR products were cloned into the pMAL-c expression vector (New England Biolabs, Beverly, MA). The plectin coordinates given in Fig. 3 are based on the numbering according to a revised rat plectin sequence (data not shown), in which a new translation start codon has been identified (1635 bp upstream of the originally published ATG; Ref. 2). pTH1 was constructed by cloning a SacI-XhoI fragment from the region from bp 1636 to 3384, which includes the coding region for two Cdk substrates (plectin and histone H1) as in vitro substrates (Fig. 1A, Cell Lysates). Unlike histone H1 (Fig. 1A, lower panels), plectin was phosphorylated by kinases contained in all three cell lysates to a similar extent (Fig. 1A, upper panels), suggesting that plectin served as substrate for these protein kinases throughout the cell cycle. Control experiments performed in the absence of exogenous plectin (Fig. 1, Control) showed that endogenous plectin was not detectable in autoradiographs. The high level of histone H1 kinase activity in mitotic cell lysates further suggested that the mitotically active Cdk, p34cdc2 kinase, was one of the major kinase activities present in these lysates. To investigate whether p34cdc2 kinase was able to phosphorylate plectin directly, the kinase was immunoprecipitated from mitotic and interphase cell lysates and its activity tested using plectin and histone H1 as substrates. The kinase immunoprecipitated from mitotic cell lysates showed a high histone H1 kinase activity, and, unlike kinases immunoprecipitated from S phase cell lysates and mock-precipitated samples, it phosphorylated plectin to a relatively high extent (~1 mol of phosphate/mol of plectin) (Fig. 1A, Immunoprecipitates; and data not shown). Samples immunoprecipitated from G₂ phase lysates also showed histone H1 and plectin kinase activities, probably due to remnants of mitotic cells in the preparation. Significant phosphorylation of both plectin and histone H1 was observed also with protein kinase preparations obtained from mitotic cell lysates by affinity purification on immobilized p13cdc2 (Fig. 1A, p13cdc2). These experiments suggested that plectin can serve as a direct in vitro substrate for mitotic p34cdc2 kinase. In contrast to immunoprecipitated samples, such activities were contained also in p34cdc2 purified kinase preparation from S phase cells, indicating that non-mitotic Cdk kinases distinct from p34cdc2 might also phosphorylate these proteins (Fig. 1A, p13cdc2). The plectin and histone H1 kinase activities of immunoprecipitated (Fig. 1B, cdc2) as well as p13cdc2-preципitated kinases (Fig. 1B, p13) were significantly reduced compared with activities contained in mitotic lysates. The effect was observed with both plectin and histone H1 as substrates.
were significantly reduced in the presence of olomoucine, an inhibitor specific for Cdk-type kinases. Activities of protein kinase A and protein kinase C were much less affected by this inhibitor (Fig. 1B, PKA and PKC), confirming that the isolated mitotic plectin kinase activity represented genuine p34\textsuperscript{cdc2} kinase.

To examine whether plectin became phosphorylated at similar sites in vivo and in vitro, two-dimensional tryptic peptide mapping was performed. Two of the spots generated from plectin immunoprecipitated from metabolically labeled mitotic CHO cells (Fig. 2, panel 3, spots a and b) were also seen in plectin phosphorylated in vitro by protein kinase A (p34\textsuperscript{cdc2}) kinase (Fig. 2, panel 4). This indicated that some of the in vivo target sites of mitotic kinases were recognized also in vitro. Peptide maps generated from purified rat glioma C\textsubscript{6} cell plectin phosphorylated with purified kinases A (Fig. 2, panel 1) or C (Fig. 2, panel 2) showed different patterns, suggesting that these kinases mainly affected plectin sites that were not phosphorylated by mitotic kinases under in vivo conditions; furthermore, mitotic cell lysates apparently did not contain any activities related to kinases A and C. Purified C\textsubscript{6} cell plectin phosphorylated by immunoprecipitated p34\textsuperscript{cdc2} kinase revealed two major peptides, a and b (Fig. 2, panel 5), both of which comigrated with the major spots generated from samples phosphorylated by mitotic extracts (Fig. 2, panel 6). This strongly suggested that plectin is a prominent target of p34\textsuperscript{cdc2} kinase contained in mitotic cell lysates. Furthermore, since these two major phosphopeptides were also present in digests of mitotic samples labeled in vivo (Fig. 2, panel 3, spots a and b; and data not shown), we concluded that purified p34\textsuperscript{cdc2} kinase phosphorylated plectin in vitro at sites, which are similar to those targeted in vivo.

Localization of the p34\textsuperscript{cdc2} Phosphorylation Sites—The consensus p34\textsuperscript{cdc2} recognition motif (S/T)-P-(X)-K/R (24) can be found twice in plectin's polypeptide chain (Ref. 2 and data not shown). One of the sites (SPAK) is located in the rod-domain, the other (TPGR) in repeat 6 of the C-terminal globular domain; in addition, repeat 6 contains a slightly degenerate motif (SPYS) (Fig. 3). To map the p34\textsuperscript{cdc2}-specific phosphorylation sites, recombinant plectin mutant proteins, corresponding to different domains of the molecule (Fig. 3), were expressed in bacteria and used as in vitro substrates for the kinase. It was found that only those mutant peptides that contained repeat 6 and/or the C-terminal tail domain were phosphorylated by p34\textsuperscript{cdc2} kinase (Figs. 3 and 4). Mutant peptides containing the N-terminal region, plectin's rod domain, or the first three repeats of the C-terminal domain were not recognized by p34\textsuperscript{cdc2} (Fig. 4 and data not shown). To address the question why the tail domain served as a good substrate for p34\textsuperscript{cdc2} kinase, even though it did not contain a consensus recognition sequence motif, two-dimensional peptide mapping was performed. Spots a and b, seen in the phosphopeptide pattern of the intact molecule after p34\textsuperscript{cdc2} phosphorylation (Fig. 5, panel 1), were missing in the peptide map derived from the mutant protein containing just the tail domain (pTH6). However, in the latter case numerous additional peptides appeared instead, which were not part of the pattern observed with the whole molecule (Fig. 5, panels 2 and 3). This suggested that the phosphorylation sites in the tail region were not accessible to the kinase in the intact molecule and therefore did not constitute native target sites. When the mutant protein encoded by pNM10 (containing repeats 4–6 and the tail) was subjected to two-dimensional peptide mapping, one of the spots appeared to
In vitro phosphorylation of plectin mutant proteins by various kinases. Truncated plectin mutant proteins encoded by the plasmids indicated were expressed in bacteria using the plM5 (pWEI1, pWEI2, pWEI3) or pMAL-c (all others) expression vector systems. Recombinant mutant proteins were subjected to phosphorylation by various kinases as described in the text. cdc2, immunoprecipitated p34\(^{\text{cdc2}}\) kinase; PKA, protein kinase A; PKC, protein kinase C. *, phosphorylation; - , no phosphorylation; nd, not determined. Numbers in scheme on top mark positions of hydroxy amino acid residues contained in potential recognition motifs for p34\(^{\text{cdc2}}\) kinase. Asterisks denote constructs modified by site-directed mutagenesis (see text).

In vitro phosphorylation of mutant proteins corresponding to N-terminal or various C-terminal repeat and tail domains. Mutant proteins encoded by pNM9 (lane 1), pTF15 (lane 2), pTH5 (lane 3), pNM1 (lane 4), pNM2 (lane 5), and pTH6 (lane 6) were expressed in E. coli, using the pMAL-c expression system. Cell lysates containing recombinant proteins, were subjected to phosphorylation using immunoprecipitated p34\(^{\text{cdc2}}\) kinase, protein kinase C, or protein kinase A, as indicated. Coomassie staining and autoradiography are shown. Arrowheads indicate expected sizes of fusion proteins; numbers, M\(_r\) \times 10\(^{-3}\).

comigrate with peptide a, seen in intact plectin, while several other spots, not seen with the whole protein (Fig. 5, panels 4 and 6), seemed to be derived from the tail domain (Fig. 5, panel 5). Since repeats 4 and 5 were not phosphorylated by p34\(^{\text{cdc2}}\) kinase (Fig. 4) and the tail showed a different pattern compared to intact plectin, the p34\(^{\text{cdc2}}\) site corresponding to spot a was likely to represent a site within repeat 6. Peptide b, derived from intact plectin, however, was not detected in the tryptic peptide maps of any of the bacterially expressed mutant proteins, which served as substrates for p34\(^{\text{cdc2}}\).

Experiments using mutant proteins representing truncated versions of repeat 6, containing either one of the two p34\(^{\text{cdc2}}\) recognition motifs identified (Fig. 3, pNM4 and pNM5), showed that only the polypeptide encoded by pNM4, containing the recognition motif TPGR, served as a target for p34\(^{\text{cdc2}}\) kinase.
p34<sup>cdc2</sup> Phosphorylation of Plectin

During interphase, 4) Mutation (Thr → Ile) of a potential p34<sup>cdc2</sup> kinase phosphorylation site within the repeat 6 domain of plectin diminished its phosphorylation by p34<sup>cdc2</sup> kinase, but not by protein kinase A (Figs. 3 and 6). 5) p34<sup>cdc2</sup> kinase prepared by affinity chromatography on p13<sup>cdc1</sup>-Sepharose or by ion exchange chromatography on DE-52 columns phosphorylated plectin at the same sites as immunoprecipitated kinase (Fig. 1 and data not shown).

Comparison of phosphopeptide maps generated from samples phosphorylated in vitro using mitotic cell lysates versus purified p34<sup>cdc2</sup> kinase suggested the major sites phosphorylated to be the same in both cases. Thus, p34<sup>cdc2</sup> kinase seems to represent the main activity among all plectin kinase activities present in mitotic cell extracts. The phosphorylation sites recognized by p34<sup>cdc2</sup> kinase in vitro are likely to represent genuine physiological targets, since the same sites were phosphorylated in vivo. The majority of phosphorylation sites affected by kinases C and A, on the other hand, were not detected in samples phosphorylated in vivo, nor in plectin phosphorylated by mitotic extracts, indicating that these kinases were not activated during the normal growth and division cycle of CHO cells.

The phosphopeptide pattern of purified plectin after p34<sup>cdc2</sup> kinase-treatment revealed two different spots (a and b), indicating two different phosphorylated sites. To map these sites, we used the bacterial expression system pMAL-c, in which recombinant peptide is expressed fused to maltose-binding protein (MBP). The relative large size of the MBP (~40 kDa) was shown to have no effect on the ability of the recombinant proteins to serve as substrates for various kinases, because proteins without MBP (after cleavage with factor Xa) behaved in the same way. Of all the different plectin domains tested, only the C-terminal part of the molecule, containing repeat 6 and/or the 3' tail domain, proved to be phosphorylated by p34<sup>cdc2</sup> kinase. When tested without the repeat 6 domain, the tail showed by far a stronger signal and became the first candidate for closer investigations. Even though it did not contain any of the reported p34<sup>cdc2</sup> consensus motifs (24), it had numerous phosphate accepting residues (21 serines and 5 threonines). However, when the phosphopeptide pattern derived from the tail was compared to that of the intact full-length protein, it turned out that none of the phosphopeptides from one source had a matching counterpart in the other. The reason why the tail, when part of the whole molecule, was not phosphorylated, despite constituting such a good in vitro substrate, probably was limited accessibility in the native molecule. This assumption was corroborated by the observation that in larger mutant proteins, containing the tail and several of the preceding repeat domains, tail-specific phosphorylation decreased and phosphopeptide patterns resembled that of the full-length protein.

The finding that repeat 6, but not the tail domain, seemed to be the natural target of p34<sup>cdc2</sup> kinase was consistent with the fact that the only perfect p34<sup>cdc2</sup> consensus sequence motif found in the C-terminal domain resided within repeat 6. Deletion and site-specific mutagenesis confirmed this site as a phosphoacceptor of p34<sup>cdc2</sup> kinase. The localization of a second phosphorylation site, suggested by the appearance of peptide b in tryptic peptide maps of intact plectin, is not clear, since none of the recombinant plectin domains, which were able to serve as substrate for p34<sup>cdc2</sup> kinase in vitro, revealed this spot in two-dimensional phosphopeptide analysis. This discrepancy could be explained in two ways. 1) There is in fact only one site and the digest of the total plectin molecule may have been incomplete, so that the second spot would represent a peptide phosphorylated at the same site but migrating to a different...
position because of its larger size. 2) The phosphorylation of the second site might be dependent on post-translational modifications of the protein, which would not occur in the bacterially expressed proteins, but could be relevant for plectin purified from rat glioma C6 cells.

The situation that a protein like plectin, containing an α-helical double-stranded coiled-coil rod domain flanked by globular domains, is preferentially phosphorylated by p34cdc2 kinase in the presumably less ordered domains adjacent to its rod applies also to the IF proteins lamin (12, 25) and vimentin (10, 26). Since their phosphorylation by p34cdc2 kinase has been implicated in the regulation of filament structure and assembly state, it remains an intriguing question to what extent plectin’s structure and functions are influenced by p34cdc2 phosphorylation.

Acknowledgments—We thank Larry Gerace (Scripps Clinic Research Institute, La Jolla, CA) for donating antiserum to p34cdc2 kinase and Heribert Hirt (University of Vienna) for providing a bacterial strain overproducing p13suc1.

REFERENCES

1. Foisner, R., and Wiche, G. (1987) J. Mol. Biol. **198**, 515–531.
2. Wiche, G., Becker, B., Luber, K., Wetzler, G., Castanon, M., Hauptmann, R., Stratowa, C., and Stewart, M. (1991) J. Cell Biol. **114**, 83–99.
3. Wiche, G. (1989) Crit. Rev. Biochem. **24**, 41–67.
4. Foisner, R., and Wiche, G. (1991) Curr. Opin. Cell Biol. **3**, 75–81.
5. Wiche, G., Gromov, D., Donovan, A., Castanon, M. J., and Fuchs, E. (1993) J. Cell Biol. **121**, 607–619.
6. Herrmann, H., and Wiche, G. (1983) J. Biol. Chem. **258**, 14610–14618.
7. Herrmann, H., and Wiche, G. (1987) J. Biol. Chem. **262**, 1320–1325.
8. Foisner, R., Traub, P., and Wiche, G. (1991) Proc. Natl. Acad. Sci. U. S. A. **88**, 3812–3816.
9. Chou, Y.-H., Bischoff, J. R., Beach, D., and Goldman, R. D. (1990) Cell **62**, 1063–1071.
10. Tsujimura, K., Ogawa, M., Takeuchi, Y., Imajoh-Ohmi, S., Ha, M., and Inagaki, M. (1994) J. Cell Biol. **126**, 31097–31106.
11. Lüscher, B., Brizuela, L., Beach, D., and Eisenmann, R. N. (1991) EMBO J. **10**, 865–875.
12. Heald, R., and Mckean, F. (1990) Cell **61**, 579–589.
13. Peter, M., Nkagawa, J., Doree, M., Labbe, J. C., and Nigg, E. A. (1990) Cell **61**, 591–602.
14. Dessev, G., Iovcheva-Dessev, C., Bischoff, J. R., Beach, D., and Goldman, R. (1991) J. Cell Biol. **112**, 523–533.
15. Terasima, T., and Tilmach, L. J. (1963) Exp. Cell Res. **30**, 350–362.
16. Laemmli, U. K. (1970) Nature **227**, 680–685.
17. Wiche, G., and Baker, M. A. (1982) Exp. Cell Res. **138**, 15–29.
18. Brizuela, L., Draetta, G., and Beach, D. (1987) EMBO J. **6**, 3507–3514.
19. Saiki, R., Gelfand, D. H., Stoffef, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, A. (1988) Science **239**, 487–491.
20. Horton, R., Hunt, H., Ho, S., Pullen, J., and Pease, L. (1989) Gene (Amst.) **77**, 61–68.
21. Simon, M., Mutzel, R., Mutzel, H., and Veron, M. (1988) Plasmid **19**, 94–102.
22. Vesely, J., Havlcek, L., Strnad, M., Blow, J. J., Donella-Deana, A., Pinna, L., Letham, D. S., Kato, J., Detivaud, L., and Leclerc, S. (1994) Eur. J. Biochem. **234**, 771–86.
23. Boyle, W. J., Van der Geer, P., and Hunter, T. (1991) Methods Enzymol. **201**, 110–149.
24. Nigg, E. (1993) Trends Cell Biol. **3**, 296–301.
25. Ward, G. E., and Kirschner, M. W. (1990) Cell **61**, 561–577.
26. Chou, Y.-H., Nigai, K.-L., and Goldman, R. D. (1991) J. Biol. Chem. **266**, 7325–7328.
Identification of Plectin as a Substrate of p34Kinase and Mapping of a Single Phosphorylation Site
Nicole Malecz, Roland Foisner, Christine Stadler and Gerhard Wiche

J. Biol. Chem. 1996, 271:8203-8208.
doi: 10.1074/jbc.271.14.8203

Access the most updated version of this article at http://www.jbc.org/content/271/14/8203

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 26 references, 8 of which can be accessed free at http://www.jbc.org/content/271/14/8203.full.html#ref-list-1