Intramolecular interactions regulate serine/threonine phosphorylation of vinculin

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Abstract **Using protein kinase C, we have studied the influence of intramolecular interactions on phosphorylation in vinculin. We show that vinculin and its 90 kDa head and 29/27 kDa tail fragments, generated by V8 proteolytic cleavage, are differentially phosphorylated. While intact vinculin and the isolated head domain are only weakly labelled, the isolated tail fragment is much more strongly phosphorylated. In the presence of the tail, the head is fully protected from the kinase. These data are consistent with our observation that native vinculin is primarily phosphorylated within the tail domain and suggest a function of vinculin phosphorylation in the regulation of the vinculin conformation.**

Key words: Vinculin; Intramolecular interaction; Protein kinase C phosphorylation; Cytoskeleton

1. Introduction

Vinculin is one of the major structural proteins located at the cytoplasmic face of microfilament-dependent cell–cell and cell–matrix adhesions of animal cells [1,2]. The 116 kDa protein consists of a large, roughly globular head domain and a smaller, rod-like tail domain of approx. 90 and 29/27 kDa, respectively. These fragments can be separated by a variety of proteinases [3,4]. Although the precise biological function of vinculin is not fully understood yet, a variety of elegant studies has shown that vinculin is important in the organisation and maintenance of cell-contact architecture. Disruption of the vinculin gene leads to embryonic death in *C. elegans* [5] and to an altered adhesion and motility in F9 cells [6,7]. Similarly, cell adhesion is disturbed in cultered fibroblasts microinjected with antibodies against vinculin [8–10]. Recent studies have shown that vinculin is a multi-ligand protein that interacts in vitro with other components of cell-contact sites, like talin, α-actinin, F-actin, paxillin and acidic phospholipids (for review see [1]). The binding of most, if not all of these ligands seems to be modulated by a head-tail interaction in the vinculin molecule itself [11–15]. Such an interaction apparently induces and stabilizes a 'closed' conformation of the molecule, resulting in inaccessibility of binding sites. However, so far, the question of how this intramolecular interaction is regulated remains open.

Reversible phosphorylation of vinculin is a potential candidate for such a master regulatory step, and indeed, vinculin has been described as a substrate of both serine/threonine and tyrosine kinases [16–18]. In cells, increased levels of phosphorylated vinculin were for example detected after activation by growth factors [19,20] and it was shown that a tissue-specific distribution of phosphorylated vinculin isoforms occurs at discrete stages in vertebrate development [21].

Here, we have investigated the correlation between vinculin's gross conformation and phosphorylation by protein kinase C, and demonstrate that the binding of the vinculin tail to the vinculin head domain greatly influences the phosphorylation of both domains.

2. Material and methods

Vinculin was purified from turkey gizzard according to Feramisco and Burridge [22]. The 90 kDa vinculin head and the 29/27 kDa tail fragments, respectively, were obtained by limited digestion of vinculin by endoproteinase Glu-C (V8 from *Staphylococcus aureus*) and purified by chromatography on MonoS as recently described [12,15]. The two tail fragments, with an apparent molecular mass of 29 and 27 kDa, respectively, have been shown to be the products of V8 cleavage at two closely spaced sites, aa850 and 857 [4]. The 29 kDa fragment is unstable and easily converts into the 27 kDa fragment. Protein concentrations were determined by quantitative amino acid analysis, according to standard procedures.

Intact vinculin and its proteolytic fragments were phosphorylated, using [γ-^{32}P]ATP and protein kinase C (PKC). The reaction mixtures (20 µl) contained 2.3 µg of vinculin or 0.46 µg of vinculin tail and/or 1.82 µg of vinculin head, respectively, in phosphorylation buffer (100 µg/ml phosphatidylserine, 20 µg/ml 1,2-dioleoyl-*sn*-glycerol (C18:1, *cis*-9), 0.027 mU PKC (Boehriger Mannheim), 20 mM Tris-acetate, pH 7.6, 10 mM NaCl, 5 mM $MgCl_2$, 0.6 mM $CaCl_2$, 0.6 mM DTE, 0.008 mM EDTA, 0.07 mM EGTA, 8 mM β-mercaptoethanol, 1 mM NaN_3). To mimic the physiological ionic strength and protein osmotic pressure, reaction mixtures were supplemented with 0.1 M KCl and 4.1% polyethylene glycol 20000 (PEG). Phosphorylation was started by the addition of 3.8 µCi [γ-^{32}P]ATP to a final concentration of 50 µM, and the mixture was incubated at 30°C for 1 h. Subsequently, the reaction was quenched by the addition of unlabelled ATP to a final concentration of 20 mM. The reaction mixtures were then either treated with immobilized V8 as described above or directly subjected to SDS-PAGE. For autoradiography, the dried gels were exposed to Kodak film (X-OMAT).

For N-terminal sequencing, purified protein was blotted onto PVDF (Immobilon P, Millipore) and N-terminally sequenced, using an Applied Biosystems gas phase sequenator (model A470) equipped with an on-line phenylthiohydantoin-amino acid analyzer.

For mass spectrometric investigations, proteins were extensively dialyzed against water and analyzed by matrix assisted laser desorption ionisation (MALDI) mass spectrometry, using a Bruker REFLEX MALD/TOF with sinapinic acid as matrix.

For the quantitative evaluation of phosphorylation, autoradiographs were scanned and incorporation was calculated with the QuantiScan program (Biosoft). Alternatively, bands were excised from the gels and radioactivity was counted directly in a Beckman liquid scintillation counter (LS6000SC).

3. Results

3.1. Differential phosphorylation of vinculin and its fragments

Identical molar amounts of vinculin, the isolated 90 kDa head and the 29/27 kDa tail domains, respectively, were sub-

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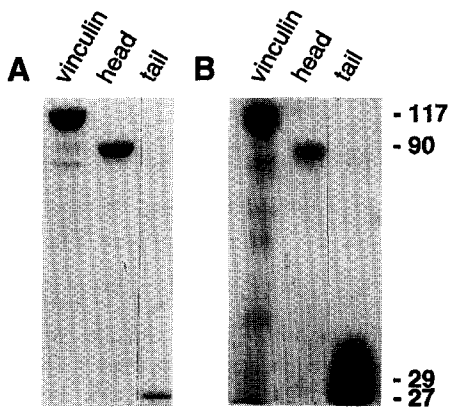

Fig. 1. Phosphorylation of vinculin and its fragments by protein kinase C. (A) Coomassie Blue stained gel and (B) the corresponding autoradiograph.

jected to in vitro phosphorylation by protein kinase C (PKC) in the presence of [γ-^{32}P]ATP. Phosphorylated samples were separated by SDS-PAGE and Coomassie Blue-stained gels were autoradiographed (Fig. 1). While the isolated tail fragment was strongly phosphorylated, genuine vinculin and the 90 kDa head domain proved relatively poor substrates, although they were also clearly labelled.

Quantitative evaluation of phosphorylation by scanning autoradiographs and counting radioactive bands derived from gels revealed that vinculin was roughly 3-times and the tail fragment approx. 25-times more strongly phosphorylated than the isolated head domain, under identical conditions (Fig. 2).

3.2. *Influence of intramolecular interactions in vinculin on phosphorylation by PKC*

The potential modulation of PKC-dependent vinculin phosphorylation by intramolecular interactions was investigated. Such interactions, mediated by a high affinity between head and tail domains have previously been described [11–15]. Equimolar mixtures of vinculin and its domains were either produced directly from genuine vinculin by V8 cleavage or reconstituted from isolated fragments. While the isolated head fragment alone was clearly labelled, in the presence of an equimolar amount of the tail, it was fully protected from

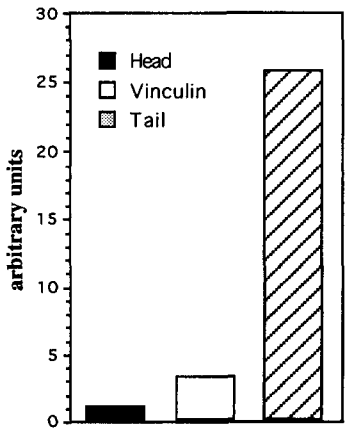

Fig. 2. Relative phosphorylation intensities of vinculin and its fragments. All values were normalized to the phosphate incorporation into the isolated head fragment.

phosphorylation (Fig. 3). In contrast, phosphorylation of the tail fragment was only moderately reduced (by approx. 20%) in the presence of stoichiometric amounts of the head fragment (not shown). To prove that binding of the tail to the head domain is responsible for this protective effect, we titrated the ratio between the two proteins. When substoichiometric amounts of vinculin tail were added to the head, phosphate was still incorporated into the head and the amount of incorporation was found to be proportional to the head-to-tail ratio (Fig. 3). The same effects were observed when the system was adapted to more physiological salt concentrations and macromolecular osmotic pressure by the addition of 0.1 M KCl and/or 4.1% PEG, although the overall phosphorylation was somewhat reduced (data not shown).

3.3. *Mapping of the phosphorylation sites*

For a topographical analysis of the phosphorylation sites, intact vinculin was phosphorylated by PKC and, after 1 h, the reaction was quenched by the addition of a 400-fold excess of unlabelled ATP. Vinculin was again clearly labelled, as seen in Fig. 4. The phosphorylated vinculin was then cleaved with V8 protease and the fragments were analyzed by SDS-PAGE and subsequent autoradiography (Fig. 4). In the particular example shown in Fig. 4, the 29 kDa tail fragment is visible in the Coomassie Blue-stained gel in addition to the 27 kDa fragment (see section 2 for explanation). Little radioactivitiy was detected in the 90 kDa vinculin head domain, while two strongly radioactive bands were seen at a position corresponding to both tail fragments. The autoradiograms also revealed an additional band at approx. 67 kDa. This fragment originates from the 90 kDa head, as it reacts with head-specific but not with tail-specific antibodies in immunoblots (not shown). It probably corresponds to a minor product of V8 cleavage, comprising aa residues 244–850, as has been described earlier [4]. For the major fragments, i.e. the 90 kDa head and the 29/27 tail domains, amino acid sequencing had shown that their N-termini are intact [4], but potential C-terminal truncation had to be excluded as a source of loss of phosphorylation

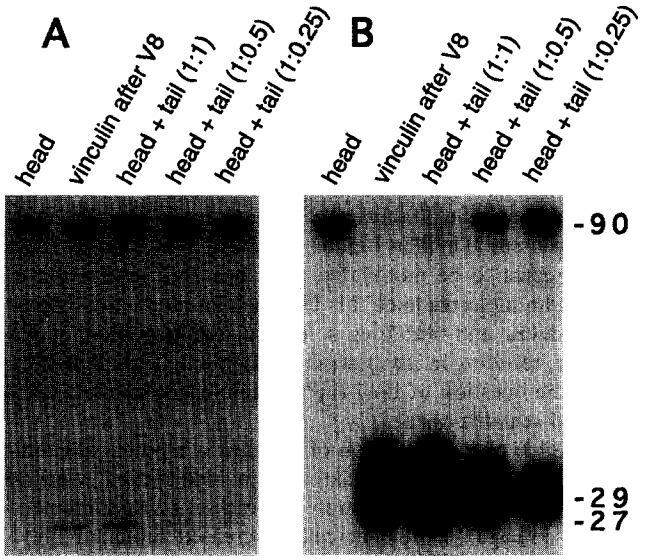

Fig. 3. Influence of head to tail interaction on phosphorylation. (A) Coomassie Blue stained gel and (B) the corresponding autoradiograph. Note that in the presence of equimolar amounts of tail fragment, the head is not phosphorylated.

sites. Therefore, we analyzed the molecular masses of both head and tail fragments, using matrix-assisted laser desorption ionization mass spectrometry (MALDI MS, Fig. 5). The masses of the head domain and the smaller tail fragment were determined to 92 675 ± 90 and 23 355 ± 25 Da, respectively. Since the N-terminal regions had been shown to be intact and the V8 cleavage sites are known [4], theoretical values for a head domain of aa2–850 and a tail of aa858–1066 were calculated from the chicken vinculin sequence. The values obtained were 92 703 and 23 336 Da, respectively. Since these figures correspond well to the MALDI-determined values, we can exclude a C-terminal truncation of both vinculin domains. Quantitative evaluation of the radioactivity incorporated into the V8 derived fragments revealed that about 60% of the label is present in the tail domain and approx. 40% in the head. Thus, the major phosphorylation sites in vinculin map to the tail domain.

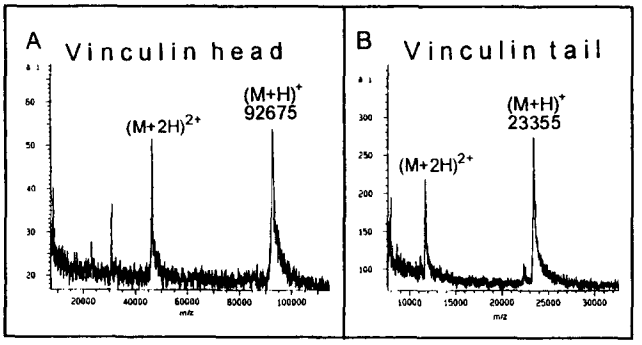

Fig. 5. Fragment characterization by mass spectrometry. (A,B) Mass spectra of the vinculin head and tail fragment, respectively. The masses corresponding to the molecular ions $(M+H)^+$ are indicated.

4. Discussion

We investigated the influence of intramolecular interactions on vinculin phosphorylation by PKC and show that the major phosphorylation sites locate to the vinculin tail domain. Mapping was based on proteolytic fragments of vinculin phosphorylated prior to cleavage, and on a comparison of the relative incorporation of phosphate into the isolated head and tail fragments. In both cases, we obtained evidence that the tail is a much better substrate of PKC than the head. This was also seen when physiological conditions were mimicked by the appropriate salt concentrations and macromolecular osmotic pressure. Using MALDI-MS and the information from N-terminal sequencing, we could exclude a possible C-terminal truncation of head or tail domains by V8 protease that had been postulated by others [23]. Our data are consistent with the view that intramolecular interactions regulate serine/threonine phosphorylation in the native vinculin molecule, since phosphorylation of the severed tail fragment is much stronger as compared with that of the tail within the intact molecule. In vinculin reconstituted from its purified head and tail domains, the head is fully protected, while the stronger phosphorylation of the tail, as compared to the genuine, intact molecule, indicates that additional sites within the tail are now exposed that have been inaccessible in the native molecule. These data add a serine/threonine kinase to the list of proteins whose access to vinculin depends on vinculin conformation [11–15,24].

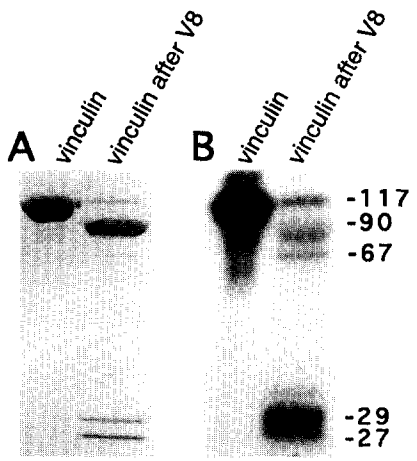

Fig. 4. Mapping of the phosphorylation site within vinculin. Vinculin was phosphorylated by PKC and then cleaved by proteinase V8. (A) Coomassie Blue stained gel and (B) the corresponding autoradiograph.

On the other hand, it is also known that protein interactions modulate vinculin conformation. In this manner, the binding of one particular partner may faciliate the binding of others. For example, it has been shown that the association of vinculin with talin, a head domain ligand, increases PKC-dependent vinculin phosphorylation [25]. Based on our findings that most phosphorylation sites are located in the tail, this can be interpreted as a talin-induced weakening of the head-tail interaction, exposing the tail phosphorylation sites to PKC. In contrast, binding of vinculin to F-actin, a tail-specific ligand [24], has the opposite effect: it decreases serine/threonine phosphorylation [25]. Presumably, in this case, F-actin and PKC compete for closely spaced or overlapping binding sites.

Since conformation and ligand binding in vinculin seem mutually dependent, phosphorylation may influence conformation as much as vice versa. On these grounds, we hypothesize that the exposure of ligand-binding sites in vinculin might be induced by reversible phosphorylation within a living cell. Such a mode of switching a structural protein's conformation and function by reversible phosphorylation is not without precedent in the literature. For instance, myosin II and the cellular src kinase also show intramolecular interactions that regulate their ligand binding affinities and catalytic properties. In both cases, this switch between an inactive and an active state is regulated by phosphorylation [26,27]. Recent data propose a function for phosphoinositol 4,5-bisphosphate (PIP_2) in the regulation of the vinculin conformation [28]. Possibly, phosphorylation and PIP_2 binding are different steps in a signal transduction cascade or exert a cooperative effect on the vinculin conformation. Although in vivo the overall phosphorylation of vinculin is low [16], it could well be a critical step in the construction and remodelling of contact sites where only a small percentage of the cellular vinculin is involved at any given time point.

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