

Identification of novel phosphorylation sites on postsynaptic density proteins

H. Jaffe^a, L. Vinade^b, A. Dosemeci^{b,c,*}

^a Protein and Peptide Sequencing Facility, NIH/NINDS, Bethesda, MD, USA

^b Laboratory of Neurobiology, NIH/NINDS, Bethesda, MD, USA

^c Program in Molecular Physiology, Marine Biological Laboratory, Woods Hole, MA, USA

Received 25 May 2004

Abstract

Phosphorylation of the components of the postsynaptic density (PSD), a protein complex lining the postsynaptic membrane, may regulate synaptic structure and function. We carried out mass spectrometric analyses to identify phosphorylation sites on PSD proteins. Phosphopeptides were isolated from the total tryptic digest of a PSD fraction by immobilized metal affinity chromatography and analyzed by liquid chromatography and tandem mass spectrometry. The phosphorylated residues detected following *in vitro* phosphorylation in the presence of Ca²⁺/calmodulin included S-1058 on SynGAP and S-1662 and S-1668 on Shank3. Other phosphorylated residues were identified in control samples, presumably reflecting phosphorylation in the intact cell. These included the homologous residues, S-295 on PSD-95 and S-365 on PSD-93, located between the PDZ2 and PDZ3 domains of these proteins; and S-367 located on the actin-binding domain of β -CaMKII. The sequence RXXSPV emerged as a common phosphorylation motif of three specialized PSD scaffolding proteins, PSD-95, PSD-93, and Shank3. Phosphorylated serine residues in several of the identified phosphorylation sites were followed by prolines, suggesting prominent involvement of proline directed kinases in the regulation of PSD components.

© 2004 Elsevier Inc. All rights reserved.

The postsynaptic density (PSD) is an electron-dense structure located underneath the postsynaptic membrane containing scaffolding molecules, receptors, and signal transduction elements. It is becoming increasingly clear that the PSD is a dynamic structure whose morphology and composition changes with activity [1–3], reflecting trafficking and reorganization of its components. These changes in the abundance and organization of PSD components as well as their functional modification are likely to be mediated, at least in part, through changes in the phosphorylation states of proteins.

Various modes of synaptic activation regulate kinases and/or phosphatases that target postsynaptic proteins. It is now well accepted that postsynaptic protein phosphorylation/dephosphorylation events regulate the traf-

fic of receptors to and from the synaptic region and are essential steps in activity-induced modification of synaptic efficacy. Indeed, many of the kinases involved in LTP and memory including Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), protein kinase A, protein kinase C, extracellular signal regulated kinase (ERK), and cyclin-dependent kinase 5 (cdk5) as well as the phosphatases implicated in LTD are thought to be localized in the PSD [4–10].

Recent evidence suggests that phosphorylation/dephosphorylation events also regulate the trafficking and turnover of scaffolding molecules that determine the organization of the PSD. For example, it has been demonstrated that NMDA-induced activation of PP2B is the initial event for the internalization and degradation of PSD-95, a protein considered to be the central organizing element of the PSD [11].

* Corresponding author. Fax: 1-301-480-1485.

E-mail address: dosemeca@mail.nih.gov (A. Dosemeci).

While studies such as the ones referred to above indicate the involvement of protein phosphorylation in post-synaptic regulation, in many instances the precise molecular mechanisms, including the identities of the phosphoproteins and/or the sites being phosphorylated, are not known. Thus, in recent years, efforts have been accelerated to identify target sites for specific kinases on key PSD proteins, as a starting point for the clarification of the functional consequences of their phosphorylation (e.g. [12,13]). By comparison, only few general proteomic studies have been carried out to uncover phosphorylated proteins and phosphorylation sites without a priori hypotheses.

Using the classical approach of 2D-electrophoresis and identification of P32-labeled spots by mass spectrometry and other means, Yoshimura et al. [14,15] compiled a list of proteins in the PSD fraction that become phosphorylated upon incubation in the presence of ATP, Ca^{2+} , and calmodulin. This early global strategy revealed that a large number of PSD proteins become phosphorylated upon activation of CaMKII and described new putative substrates for the kinase. This approach, while enabling identification of substrates of selected protein kinases, has certain shortcomings: (1) only the proteins phosphorylated *in vitro* are detected; (2) misidentification is possible due to comigration of a minor phosphorylated protein with a major nonphosphorylated protein; and (3) phosphorylated residues on the proteins are not identified.

In the present study we adopted an alternative approach, isolation of phosphorylated peptides from the total tryptic digest of the PSD fraction by IMAC and subsequent analysis by LC/MS/MS, which allows identification of phosphorylated residues. Moreover, because the protocol involves the separation of tryptic peptides rather than proteins, it is especially suited for the analysis of the PSD, whose component proteins do not separate well by 2D-electrophoresis due to solubilization problems [16]. The strategy is not based on *in vitro* P32 labeling and thus allows the detection of sites that had been phosphorylated *in vivo* and is not biased for phosphorylation sites with a high turnover. We describe analysis of a PSD fraction isolated from rat cerebral cortex and the identification of novel phosphorylated sites.

Materials and methods

Preparation of the PSD fraction and in vitro phosphorylation

The PSD fraction was prepared essentially by the method of Carlin et al. [17], with modifications as described in Dosemeci et al. [18], using frozen brains from adult Sprague–Dawley rats (collected and frozen within 2 min of sacrifice by Pel-Freez Biologicals, Rogers, AR). Protein concentration was estimated by the method of Peterson [19]. Prior to *in vitro* phosphorylation, the PSD fraction was preincubated with

0.1 M dithiothreitol for 2 h in ice. Incubation, at a final concentration of 0.4 mg PSD protein/ml, was carried out at 37°C for 20 min in the presence of 1 mM CaCl_2 , 40 $\mu\text{g/ml}$ calmodulin, 5 mM MgCl_2 , 50 $\mu\text{g/ml}$ leupeptin, 0.4 μM Microcystin-LR, and 5 μM each of the peptide inhibitors for PKC (Calbiochem, peptide 16–31) and for PKA (Calbiochem, peptide 6–22 amide) in 20 mM Hepes, pH 7.4, either in the absence (control) or presence (*in vitro* phosphorylation) of 100 μM ATP.

Phosphoproteomic analysis

Sample preparation. PSD preparation was subjected to phosphoproteomic analysis essentially according to the method of Ficarro et al. [20,21] modified as follows: PSD preparation (50 μg protein) was treated with 50 μl of 8 M urea/0.4 M NH_4HCO_3 . Proteins were reduced and alkylated by addition of 10 μl of 0.05 M DTT and heating to 55°C for 30 min followed by cooling to room temperature and addition of 10 μl of 0.1 M iodoacetamide (IAA). Alkylation was allowed to proceed for 30 min after which the sample was diluted with 130 μl water and allowed to digest with 5 μg modified trypsin (Promega, Madison, WI) overnight at 37°C. The resulting digest was diluted to 300 μl with water and acidified with TFA prior to passage through an Oasis HLB 30 mg mini column (Waters, Milford, MA) prepared by washing with MeOH followed by 0.1% TFA. Bound sample was washed with 1.5 ml of 0.1% TFA and eluted with 2 \times 500 μl of 80% CH_3CN (0.1% TFA). Eluted sample was dried in the Speed Vac (Savant, Framingham, NY). Dried sample was methylated twice as described by Ficarro et al. [21]. Methylation reaction mixture was removed in the Speed Vac and dried sample was taken up in 100 μl of 10% HOAc prior to the performance of gallium immobilized metal affinity chromatography (IMAC) utilizing a Phosphopeptide Isolation Kit (Pierce, Rockford, IL) according to manufacturer's instructions. Phosphopeptides were eluted with 3 \times 40 μl of 0.2 M Na_2HPO_4 , pH 10.5. The eluent was concentrated in the Speed Vac and its volume adjusted to 50 μl . After acidification with heptafluorobutyric acid (HFBA) the eluent was centrifuged at 20,000 g for 5 min.

Peptide synthesis. The peptide RAPS*PVKPAS*LER was synthesized by AnaSpec (San Jose, CA) and methylated as described above.

LC/MS/MS analysis. The clarified centrifugate was analyzed by LC/MS/MS on a ProteomeX integrated LC/MS proteomics workstation (ThermoFinnigan, San Jose, CA). The LC was operated in the high-throughput mode with a linear gradient of 2–65% B over 90 min where A = 0.1% HCOOH and B = CH_3CN (0.1% HCOOH) on a Bio-Basic-18 (ThermoHypersil-Keystone, Bellefonte, PA) 100 \times 0.18 mm col at 1–2 $\mu\text{l/min}$ utilizing methods and configuration provided by the manufacturer except that a 50 μl sample loop was utilized. Column loading time was increased to load the larger sample volumes. The LCQ Deca XP Plus mass spectrometer equipped with a metal spray needle (ThermoFinnigan) was operated in the “Top-Four” or “Top-Five” mode to automatically acquire (A) a full scan between m/z 300 and 1700 and (B) MS/MS spectra (normalized collision energy = 35%) of the four or five most intense ions in the full scan. Source conditions were as follows: capillary temperature, 140°C; sheath gas flow, 0 units; and ESI spray voltage, 4.2 kV. Dynamic exclusion settings were as follows: repeat count, 1 or 2; repeat duration, 0.50 min; exclusion duration, 3.00 min.

Uninterpreted MS/MS spectra were searched against the Rat/Mouse Database, which was built from the NR database (included terms: rat, mouse *Rattus norvegicus*, *Mus musculus*; excluded terms: *Arabidopsis thaliana* (Mouse Ear Cress)). Data were analyzed utilizing BioWorks 3.1 and TurboSEQUEST software (ThermoFinnigan, San Jose, CA). Search parameters were set as per Ficarro et al. [20,21]—static modifications: C = +57.0215, peptide C-terminal = +14.0269; and differential modifications: S, T, Y = +79.9799, D, E = +14.0269, M = +16.0000. The minimum ion count was set to 20 to preclude searching of poor quality spectra. As an aid in the identification of phosphopeptide MS/MS spectra, the Neutral Loss MS Detector Plot

(BioWorks 3.1) was employed to indicate those spectra that exhibit a neutral loss of phosphoric acid (98, 49 or 32.7 for the singly, doubly or triply charged precursor ions) resulting from a gas-phase β -elimination reaction. Sequence assignments were made essentially as previously described [22] based on the selection of the highest ranked phosphopeptide when (i) the second ranked peptide displayed a ΔC_n (the difference in normalized correlation score between the top scoring sequence and the next highest scoring sequence) of greater than 0.1 and (ii) the matching of a significant number (>50%) of the observed Y and B ions versus those predicted for the sequence which is manifested in a raw correlation score (X_{corr}) of >1. Confirmation of assignments was achieved by manual examination of spectra for the presence of dominant neutral loss ions equal to the number of phosphorylated residues in the sequence. When necessary, spectra were further examined for the presence of a significant number matching Y and B ions from the dehydroalanine or the dehydroamino-2-butyric acid series which result from β -elimination of H_3PO_4 from phosphorylated serine or threonine.

Results

Identification of phosphoproteins and their specific phosphorylated residues in the PSD fraction was accomplished essentially according to the method of Ficarro et al. [20,21]. PSD total protein was subjected to tryptic digestion followed by methylation of the C-termini and the aspartic and glutamic acid residues of the tryptic peptides. The resulting methylated tryptic peptides were processed by gallium IMAC to isolate phosphopeptides. Prior methylation of the tryptic digest precluded or reduced previously observed [20,21] non-specific binding of aspartic and glutamic acid-containing peptides to the IMAC column. Phosphopeptides were analyzed by LC/MS/MS for protein and phosphorylation site identification. An extended (90 min) HPLC gradient previously shown [22] to maximize the presentation of polar phosphopeptides to the mass spectrometer was utilized. The base peak chromatogram and the m/z 49 neutral loss chromatogram corresponding to an in vitro phosphorylated PSD sample are shown in Figs. 1A and B, respectively.

Analysis of the PSD digests as described above yields almost exclusively MS/MS spectra of putative phosphopeptides as evidenced by the presence of major or prominent ions corresponding to the neutral loss of the elements of phosphoric acid resulting from a gas-phase β -elimination reaction [23,24]. Few non-phosphopeptide MS/MS spectra, as evidenced by the absence of these prominent neutral loss ions, were observed, which attests to the selectivity of the method for phosphopeptide isolation. Some of these spectra might have been from phosphotyrosine peptides which do not always display these neutral loss ions [23].

Manual examination of the spectra revealed that a majority of them had few fragment ions and that many of the spectra merely displayed the neutral loss ion and little else. This well-documented problem in the mass spectrometric analysis of phosphopeptides utilizing

ion trap instruments is attributed to the inability of amide bond cleavage to effectively compete with the gas-phase β -elimination reaction [21]. Only a fraction of the spectra were judged of sufficient quality to potentially yield an ID by SEQUEST analysis (see Materials and methods for selection criteria). The rest of the spectra did not meet the set standards and were not evaluated.

All MS/MS spectra were searched in a batch mode utilizing the TurboSEQUENT program in order to identify the phosphopeptides and their specific phosphorylated residues. As an example, the MS/MS spectrum of a phosphopeptide identified as the β -CaMKII tryptic peptide (R)STVAS*MMHR is shown in Fig. 2. In addition to a dominant H_3PO_4 loss (m/z 98/2) of ion at m/z 508.4 characteristic of a doubly charged monophosphopeptide, a continuous series of Y1-1 to Y1-7 ions consistent with a phosphoserine at residue 5 were observed. Also observed were dehydroalanine series of Y and B ions, Y1-6, Y1-7, Y2-6, B1-5, B1-7, B2-9, and B2*-9 which result from fragmentation of the β -eliminated m/z 508.4 ion and can also be seen as confirmatory of a phosphoserine at residue 5.

Isolated PSDs are expected to contain a number of proteins that had been phosphorylated in intact tissue prior to cell disruption. On the other hand, many other phosphorylation sites on PSD proteins must be vacant as evidenced by the ability to incorporate P32 in vitro [7]. In the present work, PSD preparations phosphorylated in vitro as well as control samples incubated in parallel but in the absence of ATP were analyzed. The phosphorylated residues identified in control samples are presumed to be those present prior to cell disruption. Certain phosphopeptides were detected only in samples phosphorylated in vitro under conditions that favor CaMKII activity (Mg^{2+} -ATP, Ca^{2+} , calmodulin, and peptide inhibitors of PKA and PKC). Interestingly, certain other phosphopeptides were detected only in control samples. The reason for not observing these in samples incubated in the presence of ATP could be attributed to the large-scale phosphorylation of several additional proteins that would hinder the detection existing phosphorylated residues. Two independent experiments were carried out, using different PSD preparations. A list of the phosphopeptides identified in these experiments is given in Table 1, together with the names of their proteins of origin.

As expected, several phosphopeptides corresponding to CaMKII itself were identified in PSD samples incubated in the presence of Ca^{2+} , calmodulin, and ATP. These included the peptides with phosphorylated residues corresponding to T-253 and S-279 of α -CaMKII and S-280 of β -CaMKII (Table 1) which are autophosphorylation sites identified previously using different approaches [25,26]. It should be noted that not all previously identified autophosphorylation sites of CaMKII were detected

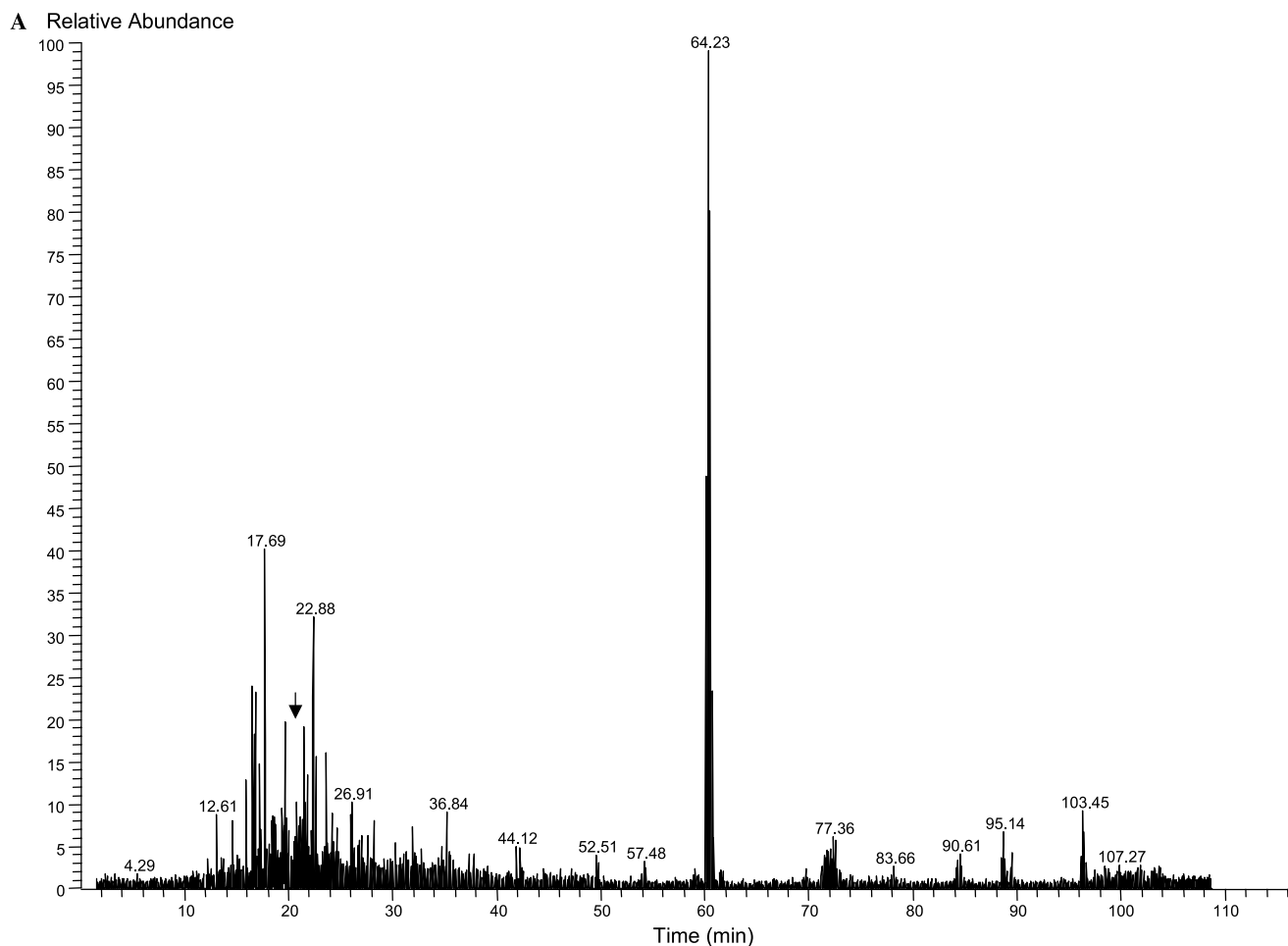


Fig. 1. LC/MS/MS analysis of phosphopeptides in the PSD fraction. The PSD fraction was phosphorylated *in vitro*, digested with trypsin, and phosphopeptides were isolated using an IMAC column before separation by HPLC. Elution of the peptide STVAS*MMHR (MS/MS in Fig. 2) at 20.25 min is indicated by an arrow. (A) Base peak chromatogram of a capillary RP-HPLC analysis of the peptide mixture by an extended linear gradient (Materials and methods). (B) Neutral loss chromatogram (m/z 49) of the peptide mixture. The generally polar phosphopeptides tended to elute early in the gradient.

by the protocol applied. Indeed, T-286 of α -CaMKII which confers autonomous kinase activity should be phosphorylated, at least partially, under the incubation conditions used as evidenced by increased immunostaining with an antibody that recognizes the T286-phosphorylated form of the protein (Dosemeci, data not shown). The failure to detect this phosphorylated residue may be due to low stoichiometry of the phosphorylation as well as signal dilution effects of differential tryptic cleavage and partial cyclization of the tryptic peptide's N-terminal glutamine to form pyroglutamate [26].

In samples phosphorylated *in vitro* in the presence of Ca^{2+} /calmodulin, we also detected the peptide (R)GKS*QQLT*VSAAQKPR with phosphorylated residues corresponding to S-1058 and T-1062 of synaptic ras GTPase activating protein (SynGAP). In the same samples, another MS/MS spectrum was tentatively identified by SEQUEST search as (R)RAPS*PVKPAS*LER originating from the scaffolding protein Shank3. Because

of some uncertainty in the identification, the above doubly phosphorylated peptide was synthesized, methylated and its MS/MS spectrum was compared to that obtained from the PSD fraction. The two spectra were essentially identical.

A phosphopeptide, (R)RYS*PVAK, was detected in all samples examined, including control samples as well as in samples phosphorylated *in vitro*. The parent protein for this peptide is identified as the major scaffolding element PSD-95. The phosphorylated residue is S-295, located between two specialized protein interaction domains of the molecule, PDZ2 (160–246) and PDZ3 (313–393). In one experiment, another phosphopeptide (R)HYS*PVECDK corresponding to the homologous protein PSD-93 was detected. The phosphorylated S-365 residue is again located between PDZ2 (193–279) and PDZ3 (421–501) domains of PSD-93.

The parent protein of a phosphopeptide detected in control samples was identified as β -CaMKII, with the

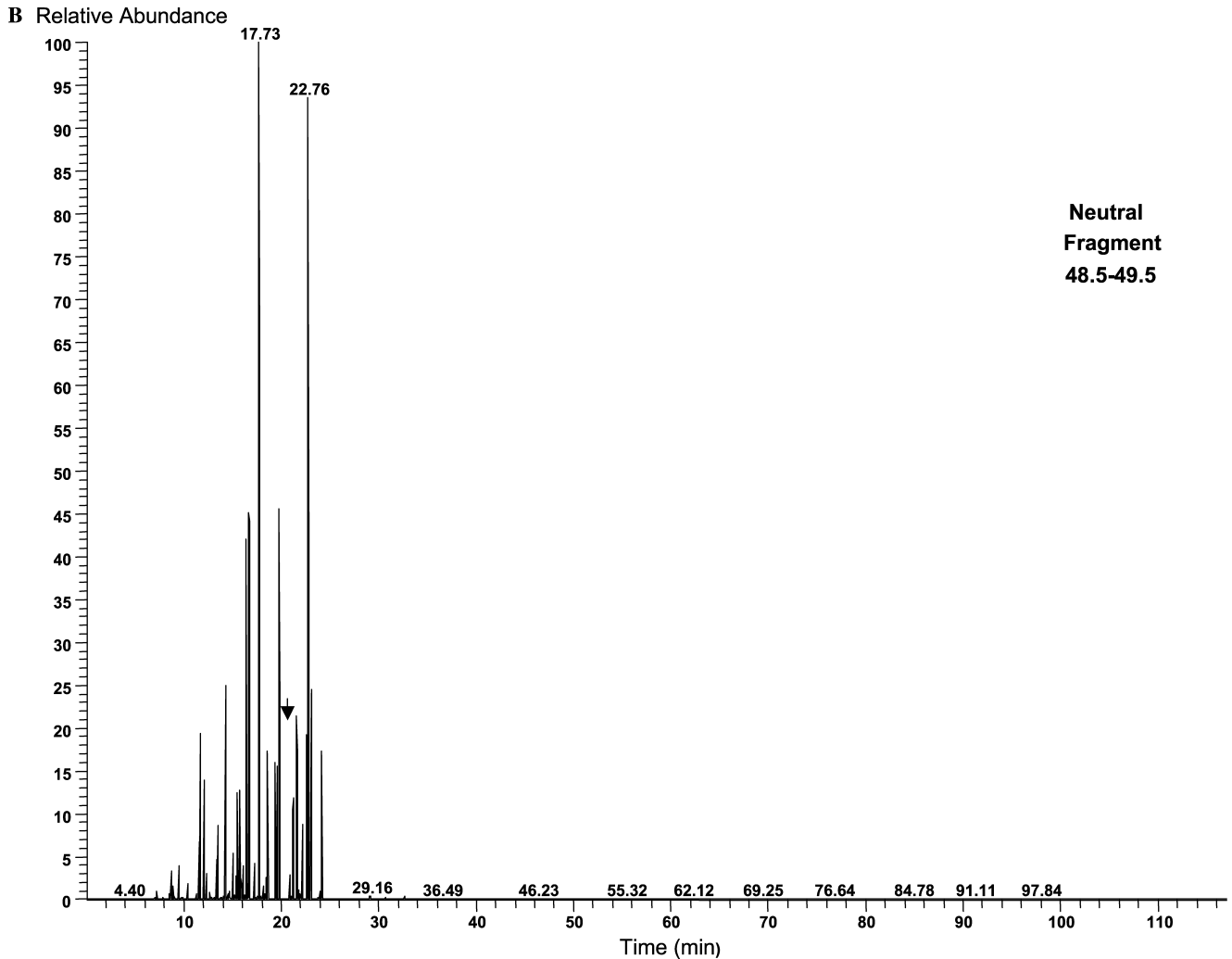


Fig 1. (continued)

phosphorylated residue corresponding to S-367 (Table 1). Two other phosphopeptides originating from the actin-interacting protein β -adducin were also detected in control PSD fractions. One of these peptides contained phosphorylated residues S-614 and S-620 and the other (one experiment) contained phosphorylated residues S-602 and S-606 (Table 1).

In addition to the phosphorylated residues on the proteins discussed above phosphopeptides corresponding to contaminants in the PSD preparation were detected including those derived from well-known phosphoproteins such as the neurofilament protein NFH, myelin basic protein, and synapsin 1b (Table 1).

Discussion

The results presented above indicate that direct mass spectrometric analysis of the total phosphopeptide fraction from a protein mixture is a rapid and convenient

method for the identification of phosphorylation sites of major component proteins in isolated organelles such as the PSD. The approach allowed identification of several new phosphorylation sites on PSD proteins. Possibly, additional sites can be uncovered upon application of alternative proteolytic reagents, as the proper size of the phosphopeptide is an important factor for its detection by the methods applied. Also, it should be noted that the strategy employed is biased for major proteins and that prior enrichment of less abundant components may be required for their detection. Finally, it is expected that selective manipulation of kinases and phosphatases would result in the phosphorylation of different sets of proteins that can subsequently be identified using the same strategy. In the present study PSD fractions phosphorylated *in vitro*, under conditions designed to activate endogenous CaMKII as well as control PSD fractions in the absence of *in vitro* phosphorylation, were analyzed. In these experiments, while an inhibitor for phosphatases of types 1 and 2A

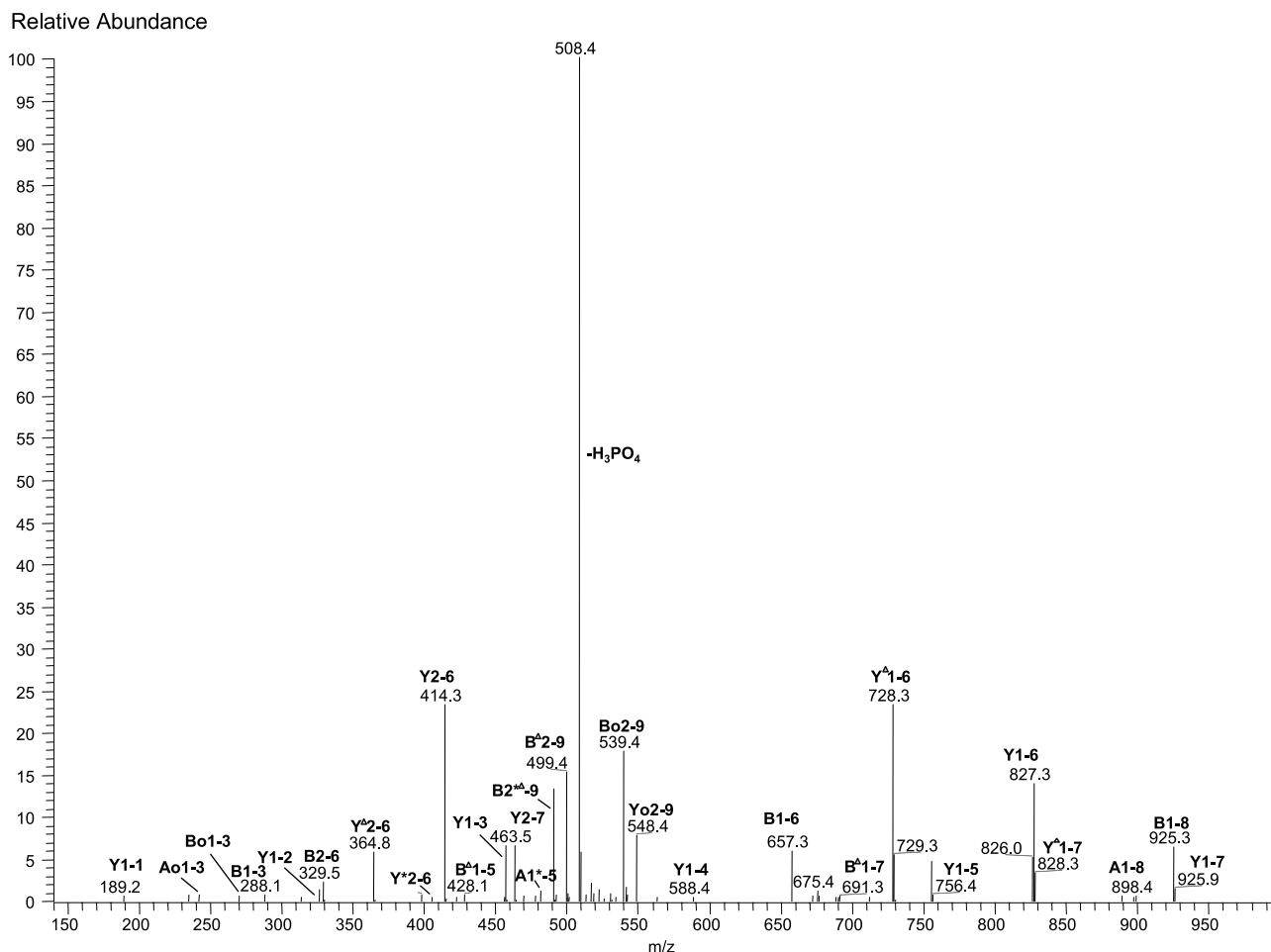


Fig. 2. Full-scan MS/MS spectrum of the doubly charged ion eluting at 21.25 min (as indicated by arrows in Fig. 1) resulting from the monophosphopeptide STVAS*MMHR. The spectrum is dominated by an ion at m/z 508.4 consistent with the neutral loss of 49 ($H_3PO_4/2$) from doubly charged parent ion at m/z 557.70. Observed A, A*, Ao, B, B*, Bo, Y, Y*, and Yo ions consistent with those predicted for STVAS*MMHR are labeled. Also labeled are the dehydroalanine series Y and B ions (indicated by a Δ), which result from fragmentation of the β -eliminated m/z 508.4 ion.

was included, no attempt was made to inhibit calcineurin activity. Inclusion of a calcineurin inhibitor may lead to the uncovering of additional phosphorylation sites.

In samples phosphorylated in vitro we identified a phosphopeptide originating from SynGAP, a prominent PSD protein [27] with phosphorylated residues corresponding to S-1058 and T-1062. The sequence around S-1058 conforms to a CaMKII consensus sequence (RXXS/T), further suggesting direct mediation by CaMKII. An article by Oh et al. [13] published shortly before the submission of this manuscript reported the phosphorylation of S-1058 as well as certain other residues upon treatment of fusion proteins containing SynGAP fragments with CaMKII and described an increase in the Ras GTPase-activation function upon phosphorylation of SynGAP by CaMKII. Our results show that S-1058 of SynGAP does indeed get phosphorylated within the PSD complex.

Also, following in vitro phosphorylation, a number of phosphopeptides corresponding to previously identified autophosphorylation sites of CaMKII were detected. In control samples (incubated without ATP) however, a unique phosphopeptide originating from β -CaMKII was observed. The phosphorylated residue on this peptide was identified as S-367 of β -CaMKII. S-367 is located on the β -specific domain of the protein and, to our knowledge, has not been described previously. It is possible that this phosphorylation site escaped detection because most previous studies focused on the autophosphorylation of purified, soluble CaMKII. Indeed, the phosphorylation of S-367 is probably mediated by a proline directed kinase, as indicated by the proline residue following the phosphorylated serine. Of the two CaMKII subunits α and β that are abundant in the PSD fraction, only β -CaMKII binds actin [28,29]. The localization of the novel phosphorylation site on the specific portion of β -CaMKII thought to

Table 1
Phosphopeptides identified in the PSD fraction

Peptide sequence	Parent protein	Residue(s)	Incubation	Number of experiments
(K)STPAS*PVQS*PTR	β -Adducin	S-602, S-606	Control	1
(K)AGTKS*PAVSPS*K	β -Adducin	S-614, S-620	Control	2
(K)MLT*INPSK	α -CaMKII	T-253	+ATP	2
(R)STVAS*CMHR	α -CaMKII	S-279	+ATP	2
(R)STVAS*MMHR	β -CaMKII	S-280	+ATP	2
(K)NSSAITS*PK	β -CaMKII	S-367	Control	2
(R)TTHYGS*LPQK	Myelin basic protein	S-70	+ATP	1
(R)TTHYGS*LPQKS*QR		S-70, S-75		1
(K)YLATAST*MDHAR	Myelin basic protein	T-21	+ATP	1
(K)YLATAST*MDHAR		S-20, T-21		1
(K)SPAEAKS*PAEAKS*PAEVK	NFH	3 repeats ^a	Control	1
(R)HYS*PVECDK	PSD-93	S-365	Control	1
(R)RYS*PVAK	PSD-95	S-295	Control	2
			+ATP	2
(R)RAPS*PVKPAS*LER	Shank 3	S-1662 S-1668	+ATP	1
(R)QAS*QAGPGPR	Synapsin Ib	S-603	+ATP	1
(R)GKS*QQLT*VSAAQKPR	SynGAP	S-1058, T-1062	+ATP	2

Two independent experiments using different PSD preparations were carried out. The last column indicates whether the phosphopeptide was detected in one (1) or both (2) experiments. PSD samples were incubated either in the absence (control) or presence (+ATP) of ATP. All phosphopeptides were identified in accordance with the standards described in Materials and methods, except for the peptide corresponding to Shank3, whose identity was confirmed using a synthetic phosphopeptide (see text). One residue N-terminal to the peptide in the parent protein is shown in parentheses. Phosphorylated residues are depicted with an asterisk (*). It should be noted that several of the phosphorylated serine residues identified are followed by prolines (underlined). The phosphorylated residues on PSD-95 and PSD-93 both fall between the PDZ2 and PDZ3 domains of the respective proteins. The phosphorylated residues on the scaffolding molecules PSD-95, PSD-93, and Shank3 are in a RXXS*PV motif.

^a The peptide sequence is repeated three times between residues 522–587 of NF-H.

confer actin binding capability [29] suggests a role in regulating the association of β -CaMKII with actin filaments.

A phosphopeptide detected in all samples examined originates from the major scaffolding protein PSD-95 and indicates phosphorylation on S-295, located between two specialized protein binding domains of the molecule, PDZ2 and PDZ3. The PDZ2 domains of PSD-95 bind NMDA receptor subunits NR2A/B, [30], shaker type K⁺ channels [31], and nitric oxide synthase [32] while PDZ3 domains bind neuroligin [33]. Thus, it is conceivable that phosphorylation of S-295 regulates the postsynaptic localization of any of these proteins. The detection of a similar phosphorylation site (S-365) on the homologous protein PSD-93, another membrane associated guanylate kinase (MAGUK) also located between PDZ2 and PDZ3 domains, consolidates and extends the finding.

Interestingly, a recent study describes NMDA-induced ubiquitination and degradation of PSD-95 that is apparently initiated through PP2B-mediated dephosphorylation [11]. Although neither the phosphorylated protein nor the phosphorylation site is known, the authors' observations imply a persistent phosphorylation that is necessary for the maintenance of synaptic PSD-95. The S-295 residue of PSD-95, found to be phosphorylated in isolated PSDs, appears to be a strong candidate for the proposed regulatory site.

CaMKII mediated phosphorylation at a different site located at the beginning of the PDZ1 domain of the *Drosophila* MAGUK, discs large protein, was described

by Koh et al. [34]. Although this site is also conserved in MAGUKs, in the present study we did not detect a corresponding phosphopeptide. However, our failure to observe phosphorylation may be due to technical reasons and cannot be considered as evidence that this specific site is not phosphorylated in mammalian MAGUKs. During the preparation of this manuscript, another group of phosphorylation sites (T19, S25, and S35) on the N-terminal domain PSD-95 has been reported [12]. Interestingly, these three residues are also followed by prolines and are target sites for the cyclin-dependent kinase 5 (cdk5).

Following in vitro phosphorylation, a phosphopeptide originating from another scaffolding molecule, Shank3, was detected. The identified phosphorylation site, S-1662 of Shank3, is located near the C-terminal "sterile α motif" (SAM) domain of the molecule. Comparison of the identified phosphopeptides corresponding to PSD-95, PSD-93, and Shank3 (Table 1) reveals that in all three proteins the phosphorylated residue is within a similar sequence which conforms to a RXXSPV motif. The observation of a similar phosphorylation motif in three major scaffolding proteins of the PSD suggests a conserved regulatory site and a common protein kinase that targets all three proteins.

Several of the phosphorylated serine residues identified are followed by proline residues, including phosphorylation sites on PSD-95, PSD-93, Shank3, β -CaMKII, and β -adducin (Table 1). Taken together, the data imply prominent involvement of proline-directed kinase(s) in

the phosphorylation of PSD proteins. The fact that most of these phosphoproteins were detected in control samples implies that they were in the phosphorylated state in the intact tissue.

In analogy to neurofilament proteins that are found to be phosphorylated on similar “SP” motifs at specific neuronal locations (axons), it is possible that the phosphorylation of such sites in the PSD is persistent. Future studies should establish whether phosphorylation on “SP” motifs is required for the targeting and/or maintenance of the proteins in the PSD. Two proline-directed kinases detected in the PSD fraction are ERK2 [8] and cdk5 [10]. ERK kinases are able to integrate multiple signals and have been implicated in the induction of stable late LTP (reviewed in [35]). Thus, an interesting possibility to be explored is the role of persistent ERK/cdk5 mediated phosphorylation of PSD proteins in the maintenance of LTP.

In conclusion the present study revealed a number of novel phosphorylation sites on PSD proteins, most notably, homologous sites on specialized scaffolding proteins with a RXXSPV motif and pointed out to a prominent role of proline directed kinases in the organization of the PSD.

Acknowledgments

We are grateful to Drs. Wayne Albers, J.-H. Tao-Cheng, and Harish Pant for a critical reading of the manuscript and to Dr. Henry Fales for their helpful discussions. This study was supported by the National Institutes of Health Intramural Program and National Science Foundation Grant 9817317 to A.D.

References

- [1] A.C. Weeks, T.L. Ivanco, J.C. Leboutillier, R.J. Racine, T.L. Petit, Sequential changes in the synaptic structural profile following long-term potentiation in the rat dentate gyrus. II. Induction/early maintenance phase, *Synapse* 36 (2000) 286–296.
- [2] A. Dosemeci, J.H. Tao-Cheng, L. Vinade, C.A. Winters, L. Pozzo-Miller, T.S. Reese, Glutamate-induced transient modification of the postsynaptic density, *Proc. Natl. Acad. Sci. USA* 98 (2001) 10428–10432.
- [3] M.D. Ehlers, Activity level controls postsynaptic composition and signaling via the ubiquitin–proteasome system, *Nat. Neurosci.* 6 (2003) 231–242.
- [4] M.B. Kennedy, M.K. Bennett, N.E. Erondy, Biochemical and immunochemical evidence that the “major postsynaptic density protein” is a subunit of a calmodulin-dependent protein kinase, *Proc. Natl. Acad. Sci. USA* 80 (1983) 7357–7361.
- [5] D.W. Carr, R.E. Stofko-Hahn, I.D. Fraser, R.D. Cone, J.D. Scott, Localization of the cAMP-dependent protein kinase to the postsynaptic densities by A-kinase anchoring proteins. Characterization of AKAP 79, *J. Biol. Chem.* 267 (1992) 16816–16823.
- [6] T. Suzuki, K. Okumura-Noji, R. Tanaka, A. Ogura, K. Nakamura, Y. Kudo, T. Tada, Characterization of protein kinase C activities in postsynaptic density fractions prepared from cerebral cortex, hippocampus, and cerebellum, *Brain Res.* 619 (1993) 69–75.
- [7] A. Dosemeci, T.S. Reese, Inhibition of endogenous phosphatase in a postsynaptic density fraction allows extensive phosphorylation of the major postsynaptic density protein, *J. Neurochem.* 61 (1993) 550–555.
- [8] T. Suzuki, K. Okumura-Noji, E. Nishida, ERK2-type mitogen-activated protein kinase (MAPK) and its substrates in postsynaptic density fractions from the rat brain, *Neurosci. Res.* 22 (1995) 277–285.
- [9] S. Strack, M.A. Barban, B.E. Wadzinski, R.J. Colbran, Differential inactivation of postsynaptic density-associated and soluble Ca²⁺/calmodulin-dependent protein kinase II by protein phosphatases 1 and 2A, *J. Neurochem.* 68 (1997) 2119–2128.
- [10] B.S. Li, M.K. Sun, L. Zhang, S. Takahashi, W. Ma, L. Vinade, A.B. Kulkarni, R.O. Brady, H.C. Pant, Regulation of NMDA receptors by cyclin-dependent kinase-5 [see comment], *Proc. Natl. Acad. Sci. USA* 98 (2001) 12742–12747.
- [11] M. Colledge, E.M. Snyder, R.A. Crozier, J.A. Soderling, Y. Jin, L.K. Langeberg, H. Lu, M.F. Bear, J.D. Scott, Ubiquitination regulates PSD-95 degradation and AMPA receptor surface expression, *Neuron* 40 (2003) 595–607.
- [12] M.A. Morabito, M. Sheng, L.H. Tsai, Cyclin-dependent kinase 5 phosphorylates the N-terminal domain of the postsynaptic density protein PSD-95 in neurons, *J. Neurosci.* 24 (2004) 865–876.
- [13] J.S. Oh, P. Manzerra, M.B. Kennedy, Regulation of the neuron-specific Ras GTPase activating protein, synGAP, by Ca²⁺/calmodulin-dependent protein kinase II, *J. Biol. Chem.* (2004) [Epub ahead of print].
- [14] Y. Yoshimura, C. Aoi, T. Yamauchi, Investigation of protein substrates of Ca(2+)/calmodulin-dependent protein kinase II translocated to the postsynaptic density, *Brain Res. Mol. Brain Res.* 81 (2000) 118–128.
- [15] Y. Yoshimura, T. Shinkawa, M. Taoka, K. Kobayashi, T. Isobe, T. Yamauchi, Identification of protein substrates of Ca(2+)/calmodulin-dependent protein kinase II in the postsynaptic density by protein sequencing and mass spectrometry, *Biochem. Biophys. Res. Commun.* 290 (2002) 948–954.
- [16] M.J. Walsh, N. Kuruc, The postsynaptic density: constituent and associated proteins characterized by electrophoresis, immunoblotting, and peptide sequencing, *J. Neurochem.* 59 (1992) 667–678.
- [17] R.K. Carlin, D.J. Grab, R.S. Cohen, P. Siekevitz, Isolation and characterization of postsynaptic densities from various brain regions: enrichment of different types of postsynaptic densities, *J. Cell Biol.* 86 (1980) 831–845.
- [18] A. Dosemeci, T.S. Reese, J. Petersen, J.H. Tao-Cheng, A novel particulate form of Ca(2+)/calmodulin-dependent [correction of Ca(2+)/CaMKII-dependent] protein kinase II in neurons, *J. Neurosci.* 20 (2000) 3076–3084.
- [19] G.L. Peterson, A simplification of the protein assay method of Lowry et al. which is more generally applicable, *Anal. Biochem.* 83 (1977) 346–356.
- [20] S.B. Ficarro, M.L. McClelland, P.T. Stukenberg, D.J. Burke, M.M. Ross, J. Shabanowitz, D.F. Hunt, F.M. White, Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*, *Nat. Biotechnol.* 20 (2002) 301–305.
- [21] S. Ficarro, O. Chertihin, V.A. Westbrook, F. White, F. Jayes, P. Kalab, J.A. Marto, J. Shabanowitz, J.C. Herr, D.F. Hunt, P.E. Visconti, Phosphoproteome analysis of capacitated human sperm. Evidence of tyrosine phosphorylation of a kinase-anchoring protein 3 and valosin-containing protein/p97 during capacitation, *J. Biol. Chem.* 278 (2003) 11579–11589.
- [22] H. Jaffe, Veeranna, H.C. Pant, Characterization of serine and threonine phosphorylation sites in beta-elimination/ethanethiol

- addition-modified proteins by electrospray tandem mass spectrometry and database searching, *Biochemistry* 37 (1998) 16211–16224.
- [23] J.P. DeGnore, J. Qin, Fragmentation of phosphopeptides in an ion trap mass spectrometer, *J. Am. Soc. Mass Spectrom.* 9 (1998) 1175–1188.
- [24] H. Jaffe, Veeranna, K.T. Shetty, H.C. Pant, Characterization of the phosphorylation sites of human high molecular weight neurofilament protein by electrospray ionization tandem mass spectrometry and database searching, *Biochemistry* 37 (1998) 3931–3940.
- [25] A. Dosemeci, N. Gollop, H. Jaffe, Identification of a major autophosphorylation site on postsynaptic density-associated Ca²⁺/calmodulin-dependent protein kinase, *J. Biol. Chem.* 269 (1994) 31330–31333.
- [26] S.G. Miller, B.L. Patton, M.B. Kennedy, Sequences of autophosphorylation sites in neuronal type II CaM kinase that control Ca²⁺-independent activity, *Neuron* 1 (1988) 593–604.
- [27] H.J. Chen, M. Rojas-Soto, A. Oguni, M.B. Kennedy, A synaptic Ras-GTPase activating protein, p135 (SynGAP) inhibited by CaM kinase II, *Neuron* 20 (1998) 895–904.
- [28] K. Shen, M.N. Teruel, K. Subramanian, T. Meyer, CaMKII β functions as an F-actin targeting module that localizes CaMKII α / β heterooligomers to dendritic spines, *Neuron* 21 (1998) 593–606.
- [29] C.C. Fink, K.U. Bayer, J.W. Myers, J.E. Ferrell Jr., H. Schulman, T. Meyer, Selective regulation of neurite extension and synapse formation by the β but not the α isoform of CaMKII, *Neuron* 39 (2003) 283–297.
- [30] H.C. Kornau, L.T. Schenker, M.B. Kennedy, P.H. Seeburg, Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95, *Science* 269 (1995) 1737–1740.
- [31] E. Kim, M. Niethammer, A. Rothschild, Y.N. Jan, M. Sheng, Clustering of Shaker-type K⁺ channels by interaction with a family of membrane-associated guanylate kinases, *Nature* 378 (1995) 85–88.
- [32] Y. Watanabe, T. Song, K. Sugimoto, M. Horii, N. Araki, H. Tokumitsu, T. Tezuka, T. Yamamoto, M. Tokuda, Post-synaptic density-95 promotes calcium/calmodulin-dependent protein kinase II-mediated Ser847 phosphorylation of neuronal nitric oxide synthase, *Biochem. J.* 372 (2003) 465–471.
- [33] M. Irie, Y. Hata, M. Takeuchi, K. Ichchenko, A. Toyoda, K. Hirao, Y. Takai, T.W. Rosahl, T.C. Sudhof, Binding of neuro-ligins to PSD-95, *Science* 277 (1997) 1511–1515.
- [34] Y.H. Koh, E. Popova, U. Thomas, L.C. Griffith, V. Budnik, Regulation of DLG localization at synapses by CaMKII-dependent phosphorylation [In Process Citation], *Cell* 98 (1999) 353–363.
- [35] J.P. Adams, J.D. Sweatt, Molecular psychology: roles for the ERK MAP kinase cascade in memory, *Annu. Rev. Pharmacol. Toxicol.* 42 (2002) 135–163.