Identification of Two Protein Kinases That Phosphorylate the Neural Cell-Adhesion Molecule, N-CAM

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The neural cell-adhesion molecule (N-CAM) is detected as at least 3 related polypeptides generated by alternative splicing of a single gene. *In vivo* the 2 larger polypeptides are phosphorylated, but the smallest polypeptide, which lacks a cytoplasmic domain, is not. We have found that the 2 larger polypeptides are phosphorylated *in vivo* on several common phosphorylation sites. Furthermore, the largest polypeptide has additional sites, suggesting that some phosphorylation occurs in that portion of the intracellular region unique to it. *In vitro* N-CAM is not a substrate for cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase I, II, or III, protein kinase C, or casein kinase II. However, we have isolated 2 protein kinases from mammalian and avian brain that phosphorylate rodent and chicken N-CAM. On the basis of their chromatographic behavior and substrate specificity, the 2 kinases are glycogen synthase kinase 3 (GSK-3) and casein kinase I (CK I). The 2 kinases phosphorylate N-CAM rapidly, to a high stoichiometry and with a low K_m for N-CAM, suggesting that the phosphorylation of N-CAM by these kinases is physiologically relevant. Both enzymes phosphorylate the 2 larger N-CAM polypeptides *in vitro* in the cytoplasmic domain on threonyl residues that are phosphorylated to a low level *in vivo*. In addition, the threonyl residues are close to seryl residues phosphorylated to a high level *in vivo*. Prior phosphorylation at the *in vivo* sites appears to be a prerequisite for phosphorylation by GSK-3 and CK I. Taken together, the results suggest that N-CAM may be physiologically phosphorylated on 2 sets of interrelated sites, one demonstrable *in vivo* and one *in vitro*. Phosphorylation on the “*in vivo*” sites is resistant to dephosphorylation and may be constitutive, while phosphorylation on the “*in vitro*” sites is much more labile.

The neural cell adhesion molecule, N-CAM, mediates cell–cell interactions throughout development and in the adult. Alterations in levels of N-CAM expression play critical roles at sites of embryonic induction (Thiery et al., 1982; Edelman, 1984, 1985). N-CAM isolated from chicken or rodent brain contains at least 3 major polypeptides with M_r values of 160,000, 130,000,

and 110,000, which have been designated the ld, sd, and ssd polypeptides, respectively (Cunningham et al., 1983; Hemperly et al., 1986b). The 3 polypeptides are generated by alternative splicing of a single gene in chicken (Murray et al., 1986a; Cunningham et al., 1987) and in mouse (Gennarini et al., 1986; Barbas et al., 1988) and differ primarily in their association with the membrane and in their cytoplasmic domains. The ssd polypeptide lacks a cytoplasmic domain (Hemperly et al., 1986b). The ld polypeptide differs from the sd polypeptide by the presence of 261 additional amino acids, which constitute the bulk of its intracellular domain (Hemperly et al., 1986a; Murray et al., 1986b).

Both the ld and sd polypeptides are phosphorylated *in vivo* (Hoffman et al., 1982; Gennarini et al., 1984; Lyles et al., 1984; Sorkin et al., 1984); ssd is not phosphorylated on amino acids (Gennarini et al., 1984). Phosphorylation occurs on both seryl and threonyl residues in chicken N-CAM (Sorkin et al., 1984) and has been reported to occur only on seryl residues in mouse N-CAM (Gennarini et al., 1984). Phosphotyrosine has never been detected. The ratios of ³²P-Ser to ³²P-Thr are different in the 2 chicken polypeptides, and there is greater incorporation of ³²PO₄ per mole into the ld species (Sorkin et al., 1984), raising the possibility that some, but not all, of the phosphorylation occurs in the additional cytoplasmic sequence.

Protein phosphorylation is an important mechanism for the regulation of protein function in many systems (Nestler and Greengard, 1984; Greengard, 1987) and as such may influence the activity of N-CAM. In particular, it may alter its surface density, binding characteristics, or interactions with other molecules at the cell surface, in the cytoskeleton or in the cytoplasm. In the current study we have further characterized the phosphorylation sites in N-CAM with respect to their locations within the ld and sd polypeptides and have shown that N-CAM is phosphorylated *in vitro* by 2 “independent” protein kinases, the kinetic properties of each being typical of physiological phosphorylation reactions. Our results also suggest that N-CAM contains 2 types of phosphorylation sites, one of which is much more resistant to dephosphorylation, and that these 2 types of phosphorylation sites may interact with each other.

Materials and Methods

Materials. Chicken, mouse, and rat N-CAM were prepared as previously described (Chuong et al., 1982; Hoffman et al., 1982). Protein phosphatase 2A, purified by the method of Resink et al. (1983), was the gift of Dr. H. C. Hemmings, Jr. Protein kinase C, purified by a modification of the method of Kikkawa et al. (1982), was the gift of Dr. K. Albert. Calcium/calmodulin-dependent protein kinase II, purified by the meth-

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od of McGuinness et al. (1985), was the gift of Dr. Y. Lai. Rabbit skeletal muscle glycogen synthase kinase-3 (GSK-3) purified by the method of Woodgett and Cohen (1984) was the gift of Dr. P. Cohen. The following proteins and enzymes were prepared by published procedures: protein phosphatase inhibitor-2 (Foulkes and Cohen, 1980), type II regulatory and catalytic subunits of cyclic AMP-dependent protein kinase (Kaczmarek et al., 1980), cyclic GMP-dependent protein kinase (Walter et al., 1980), calcium/calmodulin-dependent protein kinase I (Nairn and Greengard, 1987), calcium/calmodulin-dependent protein kinase III (Nairn et al., 1985), and calmodulin (Grand et al., 1979). Casein kinase I and II were prepared as described (Hathaway and Traugh, 1983; Hathaway et al., 1983) through the hydroxylapatite step. Casein kinase II was further chromatographed on an Ultrogel AcA44 gel-filtration column to remove contaminating casein kinase I activity.

γ - 32 P-ATP, 32 P-orthophosphoric acid, and 125 I-protein A were from New England Nuclear. Diethylaminoethyl cellulose (DE-52) was from Whatman. P-23 phosphocellulose was from Serva. Ultrogel AcA44 was from LKB. Affigel blue and HTP hydroxylapatite were from Biorad. Chymotrypsin and thermolysin were from Worthington. Trypsin and potato acid phosphatase were from Sigma. Neuraminidase was from Calbiochem. Bacterial alkaline phosphatase was from Cooper Biomedical. Phosphate-free minimum essential medium (MEM) was from Flow Laboratories.

Buffers. Buffer A consisted of 25 mM Tris (pH 7.4), 1 mM ethylenediamine tetraacetate (EDTA), 15 mM β -mercaptoethanol, 5% (vol/vol) glycerol. Buffer B consisted of 20 mM Tris (pH 7.4), 1 mM EDTA, 300 mM NaCl, 15 mM β -mercaptoethanol, 15% (vol/vol) ethylene glycol and 0.02% sodium azide. Buffer C consisted of 25 mM Tris (pH 7.5), 1 mM EDTA, 15 mM β -mercaptoethanol, and 50% (vol/vol) ethylene glycol. Buffer D consisted of 25 mM Tris (pH 7.5), 200 mM NaCl, 5 mM EDTA, 1% NP-40, 100 mM NaF, 20 mM sodium pyrophosphate, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin A, and 200 U/ml Trasylol. The pH values for these buffers refer to measurements made at 4°C.

N-CAM phosphorylation assays. Purified embryonic chicken N-CAM in solution, or bound to monoclonal anti-N-CAM coupled to Sepharose CL-2B, was used as substrate, with identical results. N-CAM was present in the assay in concentrations ranging from 0.5 to 350 μ g/ml. The reaction volume was typically 100 μ l. Calcium/calmodulin-dependent phosphorylation of N-CAM was assayed in a reaction mixture containing 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM ethyleneglycol-bis-(β -aminoethyl)N,N'-tetraacetate (EGTA), 1 mM diethioerythritol, 1.5 mM $CaCl_2$, 0.03 μ g/ml calmodulin, 50 μ M γ - 32 P-ATP (500–1500 cpm/pmol), and 0.2–0.5 μ g/ml of calcium/calmodulin-dependent protein kinase I, II, or III. Phosphorylation of N-CAM by protein kinase C was assayed with the reaction mixture described by Walaas et al. (1983), using 0.2 μ g/ml protein kinase C. Phosphorylation of N-CAM by casein kinase I and II, GSK-3, the catalytic subunit of cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase was measured in a reaction mixture containing 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate; pH 7.4), 10 mM $MgSO_4$, 1 mM EDTA, 1 mM EGTA, and 50 μ M γ - 32 P-ATP (500–1500 cpm/pmol; standard reaction mixture). Cyclic GMP (10 μ M) was present in assays using cyclic GMP-dependent protein kinase. Varying concentrations of casein kinase I and II and GSK-3 were used depending on the experiment. The concentrations of the catalytic subunit of cyclic AMP-dependent protein kinase and of cyclic GMP-dependent protein kinase were 3 and 1 μ g/ml, respectively.

The assays were initiated by the addition of γ - 32 P-ATP, and the samples were incubated at 30°C for 5 min, unless otherwise indicated. In those assays in which N-CAM in solution was used, the reaction was terminated by the addition of 20 μ l "SDS-stop solution" (Hemmings et al., 1984). The reaction mixture was heated at 90°C for 1 min and subjected to discontinuous SDS-PAGE (Laemmli and Favre, 1973). In assays where N-CAM bound to antibody coupled to Sepharose CL-2B was used, the reaction was stopped by the addition of 1 ml Buffer D and the beads were washed twice in this buffer and twice in 10 mM HEPES, pH 7.4. The beads were resuspended in 100 μ l H_2O , 20 μ l SDS-stop solution was added, and the samples were processed for SDS-PAGE as described above. The SDS-polyacrylamide gels were fixed, stained, destained, dried, and subjected to autoradiography as described by Ueda and Greengard (1977). In some experiments, the phosphorylated bands were excised from the gel and 32 P incorporation was quantitated by liquid-scintillation spectrometry.

Tissue culture. Embryonic chicken brain was cultured as previously described (Cunningham et al., 1983). Embryonic mouse tectal reagg-

gate cultures prepared by a modification of the method of Garber and Moscona (1972) were the gift of Dr. I. Shalaby and Dr. N. Rosen. Reaggregate cultures were labeled after 1, 2, or 3 weeks of growth.

Tissue labeling. For labeling with 32 P-orthophosphoric acid, dispersed brain tissue or tectal reaggregates were resuspended in phosphate-free MEM supplemented with 15 mM HEPES, pH 7.4 (1 ml/brain or 1 ml/gm reaggregate protein, respectively) containing 1 mCi/ml 32 P-orthophosphoric acid. The tissue was incubated for 12–18 hr at 37°C and then was collected by sedimentation and washed once with phosphate-free medium. The washed tissue was homogenized with a glass-Teflon homogenizer at 2100 rpm (dispersed brain tissue) or sonicated in Buffer D (reaggregate cultures). The tissue extract was clarified by centrifugation at 100,000 $\times g$ for 30 min, and the N-CAM was immunoprecipitated from the supernatant using either anti-chicken N-CAM or anti-rodent N-CAM coupled to Sepharose CL-2B (Chuong et al., 1982; Hoffman et al., 1982).

Phosphatase assays. N-CAM (1–5 μ g) was phosphorylated by GSK-3 or labeled with 32 P-orthophosphoric acid *in vivo* and immunoprecipitated, as described above. Dephosphorylation of N-CAM by protein phosphatase 2A (final concentration, 0.5 U/ml) was performed in a buffer containing 50 mM Tris (pH 7.0), 0.1 mM EDTA, 1 mM $MnCl_2$, and 15 mM β -mercaptoethanol. Dephosphorylation by potato acid phosphatase (final concentration, 0.024 U/ml) was performed in a buffer containing 50 mM piperazine-N,N'-bis [2-ethanesulfonate] (PIPES; pH 6.5), 1 mg/ml BSA, and 15 mM β -mercaptoethanol. Dephosphorylation by alkaline phosphatase (final concentration, 0.26 U/ml) was performed in a buffer containing 10 mM glycine (pH 9.8) and 1 mM $MgCl_2$. To measure dephosphorylation by endogenous embryonic chicken brain phosphatases, extracts were prepared from 9 d embryonic chicken brain using PBS containing 0.5% nonidet P-40 (NP-40) (Hoffman et al., 1982). Dephosphorylation reaction volumes were 100–500 μ l, and samples were incubated at 30–37°C for 10 min to 2 hr, as indicated, after which time the samples were subjected to SDS-PAGE and autoradiography as described above.

Miscellaneous techniques. Two-dimensional peptide mapping was performed as described by Detre et al. (1984) with the following modifications: (1) either 50 μ g/ml thermolysin or 50 μ g/ml each of both trypsin and chymotrypsin were used, as indicated, (2) ascending chromatography was performed only once using butanol:pyridine:acetic acid:water (15:10:3:12). Phosphoamino acid hydrolysis, chromatography, and detection were performed as described by Nairn and Greengard (1987). An S_2 fraction was prepared by homogenizing adult mouse brain in a 10-fold excess of PBS (Hoffman et al., 1982), centrifuging at 10,000 $\times g$ for 20 min, and collecting the supernatant. N-CAM was desialylated as described by Hoffman et al. (1982) or Cunningham et al. (1983). Protein concentrations were determined by a modification of the method of Lowry as described by Peterson (1977).

Results

Characterization of N-CAM phosphorylation in culture

The studies of N-CAM phosphorylation in culture were carried out using either chicken or mouse brain. The ld, sd, and ssd polypeptides of mouse N-CAM closely resemble those of chicken N-CAM (85% amino acid identity; Hemperly et al., 1986b; Barthels et al., 1987; Small et al., 1987; Barbas et al., 1988). Chicken N-CAM is phosphorylated on both seryl and threonyl residues (Sorkin et al., 1984), whereas mouse N-CAM has been reported to be phosphorylated only on seryl residues (Gennarini et al., 1984).

In an initial attempt to further characterize the phosphorylation sites in N-CAM, embryonic chicken brain tissue was incubated with 32 P-orthophosphoric acid and the N-CAM isolated from extracts by immunoprecipitation and analyzed by SDS-PAGE. Since embryonic N-CAM contains polysialic acid (Cunningham et al., 1983; Finne et al., 1983), the migrations of the 32 P-labeled ld and sd polypeptides were heterodisperse and overlapped. Two-dimensional thermolytic peptide mapping of the mixture of the ld and sd polypeptides revealed a complex pattern of 6 major phosphorylated peptides (Fig. 1C). Six similar ther-

Figure 1. Comparison of phosphorylation sites in the 2 major polypeptides of mouse N-CAM and in sialylated chicken N-CAM. N-CAM was labeled in culture with ^{32}P -orthophosphoric acid and isolated on anti-N-CAM-sepharose. Mouse N-CAM, digested with neuraminidase (*A* and *B*) or untreated chicken N-CAM (*C*), was detected by SDS-PAGE, excised from the gel, and subjected to 2-dimensional thermolytic peptide mapping and autoradiography as described in Materials and Methods. Electrophoresis (O, origin) was in the horizontal dimension (negative pole, *left*) and chromatography was in the vertical dimension. *A*, Autoradiogram of the thermolytic peptide map of the ld polypeptide of mouse N-CAM phosphorylated in culture. *B*, Autoradiogram of the thermolytic peptide map of the sd polypeptide of mouse N-CAM phosphorylated in culture. Peptides numbered 1–4 and 7 were common to both N-CAM polypeptides. *C*, Autoradiogram of the thermolytic peptide map of fully sialylated chicken N-CAM phosphorylated in culture.

molytic peptides were found in ^{32}P -labeled N-CAM obtained from embryonic chicken brain labeled *in ovo* (data not shown).

To compare the phosphorylation sites in the ld and sd N-CAM polypeptides, N-CAM was isolated from cultures of embryonic mouse tectal reaggregates. In these cultures, N-CAM could be labeled with ^{32}P -orthophosphoric acid to a higher specific activity than in embryonic chicken brain tissue. N-CAM was isolated by immunoprecipitation and digested with neur-

aminidase to remove the polysialic acid, and the ld and sd polypeptides were separated by SDS-PAGE. The ld and sd polypeptides were individually digested with thermolysin and 2-dimensional peptide mapping performed. Two major phosphopeptides were produced from the sd polypeptide (Fig. 1*B*, peptides 1 and 2), both of which comigrated with phosphopeptides found in the ld species (Fig. 1*A*). There were several minor phosphopeptides present in the sd polypeptide (Fig. 1*B*, peptides

Figure 2. Phosphoamino acid analysis of the 2 major polypeptides of mouse N-CAM phosphorylated in culture. The ld and sd polypeptides of mouse N-CAM phosphorylated in culture were isolated and digested with thermolysin as indicated in the legend to Figure 1. Aliquots of the total digests of the ld and sd polypeptides, or of peptides 1–4 and 4' obtained following separation of the ld digest, were hydrolyzed with 6 N HCl under vacuum for 2 hr and 1-dimensional phosphoamino acid analysis was performed. The position of unlabeled phosphoserine (*P-Ser*) and phosphothreonine (*P-Thr*) standards was determined by staining of the cellulose plate with ninhydrin. Peptides 1–4 and 7 from the sd species had identical phosphoamino acid compositions to the same peptides derived from the ld species (data not shown).

Figure 3. Phosphorylation of N-CAM by the soluble fraction of rat brain. Purified chicken N-CAM was incubated with γ - 32 P-ATP and rat brain fractions as indicated, and samples were subjected to SDS-PAGE and autoradiography. Lane 1, N-CAM alone; lane 2, N-CAM plus soluble fraction (5 μ g total protein); lane 3, N-CAM plus DEAE flow-through fraction of rat forebrain supernatant (1 μ g total protein). Aliquots assayed in lanes 2 and 3 corresponded to equal volume of the initial extract. Reaction mixtures were incubated under standard conditions for 5 min with 5 μ g N-CAM. The broad phosphorylated band of $M_r \approx 200,000$ is sialylated N-CAM (Hoffman et al., 1982). Material at the top of the gel is probably aggregated N-CAM. Molecular weights (in kDa) of standard proteins are indicated in this and all subsequent autoradiograms of SDS-polyacrylamide gels.

3, 4, and 7) that were found in the 1d polypeptide as major phosphopeptides (Fig. 1A). In addition, there were several phosphopeptides present in the 1d polypeptide that were not found in the sd polypeptide (Fig. 1A, peptides 4', 5, 6, and 8). The detection of additional phosphopeptides in the larger N-CAM species suggests that there are phosphorylation sites in the difference region, consistent with earlier results from studies of chicken N-CAM (Sorkin et al., 1984). The presence of relatively low amounts of phosphopeptides 3 and 4 in the sd polypeptide raised the possibility that some of the radioactivity in the sd region of the gel resulted from proteolytic breakdown of the 1d polypeptide. Although we cannot totally exclude this possibility, we believe it is unlikely since the addition of a cocktail of protease inhibitors to the extraction and immunoprecipitation steps had no effect on the pattern obtained.

In contrast to earlier results (Gennarini et al., 1984), phosphoamino acid analysis of mouse 1d and sd polypeptides showed that both contained phosphoserine and phosphothreonine (Fig. 2). The sd polypeptide had a higher ratio of phosphothreonine to phosphoserine consistent with the phosphoamino acid content of the individual thermolytic peptides. Only phosphothreonine was detected in peptide 1 (Fig. 2), and only phosphoserine was detected in peptides 2–8 (Fig. 2 and data not shown). In the 1d chain peptide 4' contained phosphothreonine (Fig. 2) with a trace of phosphoserine, which is not apparent from the exposure of the autoradiogram shown.

Partial purification of a protein kinase activity that phosphorylates N-CAM

In an initial attempt to characterize the protein kinase(s) responsible for the phosphorylation of N-CAM, we incubated purified chicken N-CAM with either purified protein kinases or extracts from brain. Neither native nor desialylated N-CAM were substrates for the second-messenger-regulated protein ki-

nases cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase I, II or III, or protein kinase C under our assay conditions (data not shown). A protein kinase activity that could phosphorylate purified N-CAM was identified in cytosolic brain extracts from several different species. Phosphorylation of N-CAM by brain extracts was detected by incubating purified N-CAM with γ - 32 P-ATP, Mg^{2+} , and the soluble fraction of rat (Fig. 3, lane 2), chicken or bovine brain (data not shown). N-CAM was not phosphorylated when incubated with the particulate fraction from brain of any species tested (data not shown). The kinase activity that phosphorylated N-CAM was purified from embryonic chicken, adult rat, and bovine brains, as well as from rabbit skeletal muscle, with similar results. The purification from rat brain is outlined here.

Fifty rat forebrains were homogenized and centrifuged at $150,000 \times g$ for 45 min (McGuinness et al., 1985), and the soluble fraction was applied to a DEAE-cellulose column (5×10 cm) equilibrated with Buffer A. The flow-through contained significantly more than 100% of the applied N-CAM kinase activity (Fig. 3, lane 3), suggesting the presence of an inhibitor of this activity in the soluble fraction which was retained by the DEAE-cellulose column. Material in the flow-through was applied to a phosphocellulose column (5×5 cm) equilibrated with Buffer A. The column was washed with 2 volumes of Buffer A and eluted with a linear gradient of 0–0.3 M NaCl; N-CAM kinase activity eluted with a peak at 0.08 M NaCl (Fig. 4). The peak fractions were pooled, and solid ammonium sulfate was added to 65% saturation. After centrifugation at $10,000 \times g$ for 30 min, the pellet (containing >95% of kinase activity) was resuspended in Buffer B and applied to an Ultrogel AcA44 gel filtration column (2.5×80 cm). N-CAM kinase activity eluted with an apparent M_r of $\approx 50,000$ (Fig. 5). The active fractions were pooled and applied to a column of Affigel blue (2×10

Figure 4. Chromatography of N-CAM kinase activity on phosphocellulose. The flow-through from a DEAE-cellulose column was applied directly to a phosphocellulose column (5 × 5 cm) equilibrated with Buffer A. The column was washed with Buffer A and eluted with a linear gradient of 0–0.3 M NaCl in Buffer A. The flow rate was 100 ml/hr, and 5 ml fractions were collected. Aliquots (5 μ l) of every fifth fraction were assayed for N-CAM kinase activity (cpm ³²P incorporated into purified N-CAM was determined by SDS-PAGE and liquid-scintillation spectrometry) using the standard assay conditions.

cm) equilibrated with Buffer B. The column was eluted with a linear gradient of 0.05–2.0 M NaCl; N-CAM kinase activity eluted with a broad peak at 1.0–1.2 M NaCl (Fig. 6). Active fractions that contained only one kinase activity based on substrate specificity studies (see below) were pooled, concentrated against dry sucrose, dialyzed against Buffer C, and stored at –20°C until use.

Comparison of N-CAM kinase with glycogen synthase kinase-3

The N-CAM kinase activity purified as described above was not regulated by any of the known second messengers, for example, cAMP or Ca²⁺. Comparison of the properties of the kinase activity with those of known “independent” kinases re-

Figure 5. Gel filtration of N-CAM kinase activity. The peak fractions from the phosphocellulose column were pooled, precipitated with ammonium sulfate at 65% saturation, and the resuspended pellet applied to an Ultrogel AcA44 column (2.5 × 80 cm) equilibrated with Buffer B. The flow rate was 20 ml/hr and 2.5 ml fractions were collected. Aliquots (10 μ l) were assayed for N-CAM kinase activity using the standard assay. Arrows indicate the positions of elution of blue dextran (void volume), and the molecular-weight standards, lactate dehydrogenase (140,000), transferrin (80,000), BSA (68,000), and ovalbumin (45,000).

Figure 6. Chromatography of N-CAM kinase activity on Affigel blue. The peak fractions from the gel-filtration column were pooled and applied to an Affigel blue column (2 × 10 cm) equilibrated with Buffer B, washed with 0.3 M NaCl in Buffer B, and eluted with a linear gradient of 0.05–2 M NaCl in Buffer B. The flow rate was 20 ml/hr, and 1 ml fractions were collected. Aliquots (5 μ l) were assayed for N-CAM kinase activity using the standard assay.

Figure 7. Phosphopeptide mapping of glycogen synthase phosphorylated by N-CAM kinase and by GSK-3. Rabbit muscle glycogen synthase was phosphorylated by (A) the partially purified N-CAM kinase described above or (B) GSK-3 purified from rabbit skeletal muscle. Two-dimensional phosphopeptide mapping was performed as described in Figure 1 except that trypsin plus chymotrypsin replaced thermolysin.

vealed a striking similarity with GSK-3 in chromatographic behavior on DEAE-cellulose, phosphocellulose, gel filtration, and hydroxylapatite. GSK-3 is one of several protein kinases that phosphorylate glycogen synthase and has been purified from rabbit skeletal muscle using glycogen synthase as a substrate (Rylatt et al., 1980; Hemmings et al., 1982a; Woodgett and Cohen, 1984). Comparison of the 2-dimensional tryptic/chymotryptic phosphopeptide maps of glycogen synthase phosphorylated by the kinase that phosphorylates N-CAM, purified as described above (Fig. 7A) and by authentic rabbit skeletal muscle GSK-3 (Woodgett and Cohen, 1984) (Fig. 7B) indicated that the 2 kinases phosphorylated the same peptide.

In addition to glycogen synthase, there are only 2 other well-characterized substrates for GSK-3, the type II regulatory subunit of cyclic AMP-dependent protein kinase (R_{II}) and phosphatase inhibitor-2 (I-2) (Hemmings et al., 1982b, c). The kinase from brain phosphorylated both of these substrates at rates comparable to N-CAM and glycogen synthase (see below). In ad-

dition, the kinase that phosphorylated N-CAM coeluted on CM-cellulose with the kinase that phosphorylated R_{II} and I-2, suggesting that all 3 proteins were substrates for the same protein kinase. Thus, the protein kinase partially purified from brain using N-CAM as a substrate is very similar to rabbit skeletal muscle GSK-3 on the basis of its chromatographic properties, sites of glycogen synthase phosphorylation, and substrate specificity. Furthermore, authentic rabbit skeletal muscle GSK-3 phosphorylated purified N-CAM at rates comparable to the enzyme purified from chicken brain (data not shown). We therefore refer to this protein kinase as GSK-3.

Phosphorylation of the N-CAM polypeptides by GSK-3 and casein kinase I and II

The phosphorylation of the ld and sd polypeptides of N-CAM by GSK-3 and by 2 other "independent" protein kinases, namely, casein kinases I and II, was investigated in more detail. Embryonic chicken N-CAM was phosphorylated by GSK-3, the samples incubated either without or with neuraminidase, and resolved by SDS-PAGE. GSK-3 phosphorylated both the ld and sd polypeptides, with the ld polypeptide being phosphorylated to a greater extent (Fig. 8A). Both the ld and sd polypeptides were also phosphorylated by casein kinase I (CK I; Fig. 8B) but not by CK II. In these experiments the mass ratio of $M_r = 170,000$ to $M_r = 140,000$ was about 0.9:1.0, and the proportion of ^{32}P incorporated into the 2 polypeptides was about 5:1; thus, the larger polypeptide was phosphorylated by both enzymes to a greater extent than was the smaller. The third polypeptide of N-CAM, the ssd polypeptide, which contains no cytoplasmic domain, was not a substrate for GSK-3, CK I, or CK II. Mouse and rat N-CAM contain ld, sd, and ssd polypeptides similar to those of chicken N-CAM. The phosphorylation of rat N-CAM

Table 1. Relative rates of phosphorylation of N-CAM and glycogen synthase by GSK-3 partially purified from rat brain, and of N-CAM and casein by CK I partially purified from rat brain

	N-CAM	Glycogen synthase	Casein
GSK-3	2.96 ± 0.28^a	4.56 ± 0.60^a	N.D.
CK I	169 ± 4^b	N.D.	94 ± 1^b

Substrate concentrations were $1.25 \mu M$ for GSK-3 assays and $1.0 \mu M$ for CK I assays.

N.D., not determined.

^a nmol/mg/min.

^b pmol/ml/min.

Figure 8. Phosphorylation of chicken and rodent N-CAM by partially purified GSK-3 and CK I. Native (N'ase -) or neuraminidase-digested (N'ase +) N-CAM bound to anti-N-CAM coupled to Sepharose CL-2B was phosphorylated by either GSK-3 or CK I using the standard assay conditions and samples were separated by SDS-PAGE and autoradiography performed. *A*, Embryonic chicken N-CAM phosphorylated by GSK-3. *B*, Embryonic chicken N-CAM phosphorylated by CK I. *C*, Adult rat N-CAM phosphorylated by GSK-3 and CK I. Heavy arrow, ld polypeptide; light arrow, sd polypeptide. The band migrating at $M_r \approx 55,000$ is monomeric immunoglobulin heavy chain.

was similar to that of chicken N-CAM, with GSK-3 and CK I phosphorylating the ld and, to a lesser extent, the sd polypeptides (Fig. 8C). Rat N-CAM was not phosphorylated by cyclic AMP-dependent protein kinase, cyclic GMP-dependent protein kinase, calcium/calmodulin-dependent protein kinase I, II or III, protein kinase C, or CK II (data not shown).

Kinetics of phosphorylation of N-CAM by GSK-3 and CK I

If N-CAM is a physiological substrate for GSK-3 or CK I, it should be phosphorylated rapidly, with a low K_m and to a high stoichiometry. The rate of phosphorylation of N-CAM by GSK-3 was 65% that of glycogen synthase (Table 1). The rate of phosphorylation of N-CAM by CK I was 180% that of casein. Analysis of the phosphorylation of N-CAM by partially purified GSK-3 using a Lineweaver-Burk plot gave an approximate K_m of 24 μ M and a V_{max} of 120 nmol/mg/min (not shown). Purified N-CAM (0.5 μ mol) was incubated with GSK-3 using the standard assay for various periods of time. The phosphorylation of N-CAM was essentially complete after 30 min (not shown). Assuming an average molecular weight of 135,000 for N-CAM, the maximum stoichiometry of phosphorylation by GSK-3 was calculated to be 0.9 mol/mol.

Characterization of N-CAM phosphorylation sites

Two-dimensional thermolytic peptide mapping of chicken N-CAM showed that GSK-3 (Fig. 9A) and CK I (Fig. 9B) both phosphorylated N-CAM on the same peptide. Threonine was the major (>95%) amino acid phosphorylated by either kinase (Fig. 9C). Similar results were obtained from phosphopeptide mapping and phosphoamino acid analysis of rat N-CAM phosphorylated by GSK-3 or CK I (data not shown).

Most protein kinases act intracellularly, and the observation that the sd polypeptide was not a substrate for GSK-3 or CK I, suggested that the N-CAM phosphorylation site is intracellular. To investigate whether CK I and GSK-3 phosphorylated N-CAM on its intracellular domain, right-side-out embryonic chicken brain vesicles (Hoffman and Edelman, 1983) were incubated with CK I and γ - 32 P-ATP. No significant phosphorylation of either the ld or sd polypeptides of N-CAM was observed when CK I was added in the absence of NP-40 (Fig. 10). However, when 0.5% NP-40 was present during the reaction, both N-CAM species were phosphorylated by CK I. In contrast, there was no effect of NP-40 on the phosphorylation of purified N-CAM by GSK-3 or CK I. Some N-CAM phosphorylation

Figure 9. Phosphopeptide mapping and phosphoamino acid analysis of chicken N-CAM phosphorylated *in vitro*. Two-dimensional phosphopeptide mapping was performed as described in the legend to Figure 1. *A*, Autoradiogram of the thermolytic peptide map of chicken N-CAM phosphorylated by GSK-3. *B*, Autoradiogram of the thermolytic peptide map of chicken N-CAM phosphorylated by CK I. *C*, Autoradiogram showing the amino acids phosphorylated in chicken N-CAM by GSK-3 (lane 1) and by CK I (lane 2). The positions of phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) were detected by staining with ninhydrin, and radioactivity was quantitated by liquid-scintillation counting. The arrow indicates the position of incompletely hydrolyzed phosphopeptides.

Figure 10. Phosphorylation of N-CAM in chicken brain membrane vesicles. Vesicles were incubated in the standard phosphorylation reaction mixture made isotonic with sucrose and containing the following additions: lane 1, no additions; lane 2, NP-40 alone; lane 3, CK I alone; lane 4, NP-40 and CK I. N-CAM was immunoprecipitated from NP-40-solubilized vesicles and digested with neuraminidase, and the samples were subjected to SDS-PAGE and autoradiography. Heavy arrow, ld polypeptide; light arrow, sd polypeptide.

Figure 11. Phosphopeptide mapping of mouse N-CAM phosphorylated first *in vivo* and then by GSK-3 *in vitro*. Two-dimensional phosphopeptide mapping was performed as described in Figure 1. *A*, Autoradiogram of the thermolytic peptide map of embryonic mouse brain ld polypeptide of N-CAM, phosphorylated in culture. *B*, Autoradiogram of the thermolytic peptide map of embryonic mouse brain ld polypeptide of N-CAM phosphorylated first in culture followed by phosphorylation with γ - ^{32}P -ATP and GSK-3. Peptides 3' and 4' indicate the positions of the peptides phosphorylated *in vivo* which are additionally phosphorylated by GSK-3 *in vitro*. Note that because of the higher specific activity of the peptides phosphorylated by GSK-3, the autoradiogram in *B* was exposed for less time than that in *A*. *C*, Autoradiogram of a mixture of equal amounts of the thermolytic digests shown in *A* and *B*. *D*, Autoradiogram indicating the amino acids phosphorylated in peptides 3' and 4' obtained from the samples separated as shown in *B*.

was seen upon the addition of NP-40 alone to the chicken brain vesicles. Phosphorylation by this endogenous N-CAM kinase activity occurred on the same thermolytic peptide phosphorylated by GSK-3 and CK I (data not shown).

GSK-3 phosphorylation sites are usually close to other phosphorylation sites, with which they interact (e.g., Woodgett and Cohen, 1984). To determine whether this was the case for N-CAM, mouse N-CAM was labeled in culture with ^{32}P -orthophosphoric acid and isolated by immunoprecipitation. An aliquot was then further phosphorylated with GSK-3 and γ - ^{32}P -ATP, and both samples were digested with neuraminidase and the polypeptides separated by SDS-PAGE. Comparison of the thermolytic phosphopeptide map of the ld polypeptide of N-CAM phosphorylated in culture (Fig. 11*A*), with that of the ld polypeptide phosphorylated first in culture and then by GSK-3 (Fig. 11*B*), revealed that the migration of phosphopeptides 3 and 4 were markedly changed following phosphorylation by GSK-3. Note that because of the higher specific activity of the peptides phosphorylated by GSK-3, the autoradiogram shown in Figure 11*B* was exposed for a shorter time than that in Figure 11*A*. Similar changes in the positions of peptides 3 and 4 were seen in the sd polypeptide (data not shown), where they were present in lower amounts than in the ld polypeptide (see Fig. 1). Analysis of the data shown in Figure 11, as well as results from additional experiments in which peptides 3/3' were present in lower amounts than peptides 4/4', showed that peptide 3 phosphorylated on serine in culture (Fig. 2) was further phosphorylated by GSK-3 on threonine (Fig. 11*D*) and migrated to

the position indicated (3'). Similar analysis as well as mixing experiments (Fig. 11*C*) showed that peptide 4, phosphorylated on serine in culture, was further phosphorylated by GSK-3 on threonine (Fig. 11*D*). It then migrated to the same position as peptide 4', which was labeled in low amounts in culture (see Fig. 1*A*). The electrophoretic and chromatographic properties of peptide 4' phosphorylated either in culture or following phosphorylation in culture and by GSK-3 were the same as those of the peptide phosphorylated in purified N-CAM by GSK-3 or CK I (Fig. 9).

Phosphatase sensitivity of N-CAM labeled by GSK-3 or in culture

To further compare the GSK-3/CK I and the *in vivo* phosphorylation sites, N-CAM phosphorylated under both conditions was incubated with protein phosphatase 2A, a protein phosphatase with a broad substrate specificity (Ingebritsen and Cohen, 1983). When N-CAM phosphorylated by GSK-3 was incubated with protein phosphatase 2A for 45 min, essentially all of the ^{32}P was removed (Fig. 12*A*). In contrast, after a similar incubation with phosphatase 2A, chicken N-CAM labeled with ^{32}P -orthophosphoric acid in culture remained fully phosphorylated (Fig. 12*B*).

As the GSK-3/CK I site is readily dephosphorylated *in vitro*, it is likely that during the isolation of N-CAM this site is dephosphorylated by endogenous phosphatases. Indeed, mammalian brain contains high levels of protein phosphatase 2A activity (Ingebritsen et al., 1983). To determine whether chicken brain contains a similar phosphatase activity, N-CAM labeled

Figure 12. Dephosphorylation of N-CAM. N-CAM was phosphorylated either *in vitro* or *in vivo* and dephosphorylated as indicated, and samples were subjected to SDS-PAGE and autoradiography. Purified chicken N-CAM was phosphorylated using GSK-3 and γ -³²P-ATP (A) or phosphorylated with ³²P-orthophosphoric acid in culture (B), and in both cases incubated without (lane 1) or with (lane 2) protein phosphatase 2A (0.5 U/ml) for 45 min. C, N-CAM was phosphorylated using GSK-3 and γ -³²P-ATP and incubated without (lane 1) or with (lane 2) an NP-40 extract of embryonic chicken brain for 10 min. D, Autoradiogram of an immunoblot of unlabeled N-CAM incubated without (lane 1) or with (lane 2) NP-40 extract of embryonic chicken brain. N-CAM was detected by incubation of the blot with a 1:10,000 dilution of rabbit anti-chicken N-CAM antiserum followed by ¹²⁵I-protein A.

by GSK-3 was incubated with NP-40 extracts of embryonic brain. Eighty-nine percent of the phosphate incorporated into N-CAM by GSK-3 was removed after 10 min of incubation at 37°C (Fig. 12C). This incubation did not result in significant proteolysis of N-CAM (Fig. 12D). Thus, chicken brain, as well as mammalian brain, contains a phosphatase that readily dephosphorylates the GSK-3/CK I site in N-CAM.

All other well-characterized substrates for GSK-3 are phosphorylated only after prior phosphorylation by CK II (Hemmings et al., 1982b; Picton et al., 1982; DePaoli-Roach, 1984). The failure to phosphorylate N-CAM using CK II and the ability of the protein to be phosphorylated by GSK-3 might thus be due to the site(s) for CK II (or another kinase) being fully phosphorylated in isolated N-CAM. The CK II sites in both R_{II} and glycogen synthase retain significant amounts of phosphate during purification as these sites are poor substrates for endogenous protein phosphatases. They are, however, excellent substrates for potato acid phosphatase (Hemmings et al., 1982b; Picton et al., 1982).

The stability of the phosphate incorporated into N-CAM *in vivo* was therefore investigated by comparing the abilities of phosphatase 2A, potato acid phosphatase, and alkaline phosphatase to dephosphorylate mouse N-CAM (Fig. 13A). Potato acid phosphatase removed greater than 80% of the ³²P from N-CAM labeled in culture, while alkaline phosphatase was less effective and, as with chicken N-CAM, phosphatase 2A had little or no effect. Incubation with potato acid phosphatase did not result in appreciable proteolysis of N-CAM (Fig. 13B).

To investigate the relationship between the sites phosphorylated by GSK-3 and the sites dephosphorylated by potato acid

phosphatase, purified chicken N-CAM was treated with potato acid phosphatase, the phosphatase removed by washing, and the N-CAM then incubated with either GSK-3 or CK II or CK I followed by GSK-3 (Fig. 14). Dephosphorylation with acid phosphatase converted N-CAM to a form that was poorly phosphorylated by either GSK-3 (Fig. 14, lane 2) or CK I (data not shown). N-CAM was not a substrate for CK II despite the acid phosphatase treatment (Fig. 14, lane 3). Furthermore, incubation of dephosphorylated N-CAM with CK II did not convert the protein to a form that was readily phosphorylated by GSK-3 (Fig. 14, lane 4). In addition, after dephosphorylation by acid phosphatase, N-CAM was not phosphorylated by mouse brain extracts (Fig. 14, lanes 5 and 6) or by any of the cyclic nucleotide-dependent protein kinases used previously (data not shown). These results suggest that prior phosphorylation on the *in vivo* sites is a prerequisite for phosphorylation by GSK-3 and CK I.

Discussion

In the current work we have examined the phosphorylation of N-CAM both *in vivo* and *in vitro*. The results indicate that differential phosphorylation of the α and β polypeptides occurs *in vivo* and that 2 previously described protein kinases, GSK-3 and CK I, phosphorylate both chains *in vitro*. Both GSK-3 and CK I are "independent" protein kinases, that is, they have no known physiological activators. GSK-3 phosphorylates 3 other substrates in addition to N-CAM; these are glycogen synthase, inhibitor-2 (Hemmings et al., 1982c), and the regulatory subunit of type II cyclic AMP-dependent protein kinase (Hemmings et al., 1982b). In contrast, CK I phosphorylates a large number of diverse substrates (Hathaway and Traugh, 1982). N-CAM was

Figure 13. Dephosphorylation of N-CAM by potato acid phosphatase. *A*, N-CAM was phosphorylated in culture and dephosphorylated as indicated, and samples were subjected to SDS-PAGE and autoradiography. Lane 1, no phosphatase; lane 2, protein phosphatase 2A (0.5 U/ml for 45 min); lane 3, alkaline phosphatase (0.26 U/ml for 2 hr); and lane 4, potato acid phosphatase (0.024 U/ml for 2 hr). *B*, Autoradiogram of an immunoblot of N-CAM incubated without or with acid phosphatase as above, subjected to SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. N-CAM was detected by incubation of the nitrocellulose with a 1:10,000 dilution of rabbit anti-mouse N-CAM antiserum followed by ^{125}I -protein A. The arrow indicates the position of N-CAM.

found not to be phosphorylated *in vitro* by any of the well-characterized calcium-dependent or cyclic nucleotide-dependent protein kinases.

Two distinct types of N-CAM phosphorylation sites were distinguished by their susceptibility to phosphorylation and dephosphorylation. The major sites phosphorylated in culture were resistant to dephosphorylation by protein phosphatase 2A, while the major site labeled by GSK-3 or CK I *in vitro* (but detected only at low levels in culture) was quite sensitive. These differences suggest the presence of both relatively stable and transient types of phosphorylation of N-CAM, each of which might modulate N-CAM function in different ways.

The 2 types of N-CAM phosphorylation sites apparently interact. N-CAM is poorly phosphorylated by GSK-3 if the sites phosphorylated in culture are first dephosphorylated by potato acid phosphatase. The other known substrates of GSK-3, glycogen synthase, R₁₁, and I-2, show similar behavior in that each must be phosphorylated by CK II before it can be phosphorylated by GSK-3 (Hemmings et al., 1982b; Picton et al., 1982; DePaoli-Roach, 1984).

Although N-CAM labeled in culture is phosphorylated most prominently on sites not phosphorylated by GSK-3 or CK I, the data suggest that N-CAM is a physiological substrate for GSK-3 and CK I. Peptide 4', the major peptide phosphorylated by GSK-3 is phosphorylated to a small but measurable extent in N-CAM labeled in culture. N-CAM is phosphorylated by GSK-3 and CK I at rates comparable to those for glycogen

synthase and casein, respectively (Table 1), as expected for a physiological substrate (Krebs and Beavo, 1979). A kinetic analysis of the phosphorylation of N-CAM by GSK-3 gave K_m and V_{max} values similar to those obtained for physiological substrates of other protein kinases. In addition, GSK-3 phosphorylates N-CAM with a stoichiometry of approximately 1 mol/mol, consistent with N-CAM being a specific substrate for GSK-3, which has previously been found to phosphorylate only 3 other proteins stoichiometrically. Furthermore, GSK-3 and CK I phosphorylate threonyl residues in similar thermolytic peptides in chicken, mouse, and rat N-CAM, suggesting that functional constraints favor the conservation of this site.

There are a number of possible reasons for the low level of phosphorylation of N-CAM on the GSK-3/CK I site in cells labeled in culture. This site may be phosphorylated in the cultured tissue but dephosphorylated by endogenous protein phosphatases during isolation (see Fig. 12), despite the presence of phosphatase inhibitors. Alternatively, *in vivo* this site may only be phosphorylated in response to a specific stimulus.

There are several explanations of why the phosphorylation of the major *in vivo* sites were not detected using *in vitro* assays. The sites phosphorylated *in vivo* are most likely stoichiometrically phosphorylated, a possibility favored by the observations that much of the phosphate incorporated in culture is resistant to removal by endogenous protein phosphatases (Figs. 12, 13) and that purified N-CAM contains about 3 mol phosphate/mol protein (Hoffman et al., 1982). Moreover, peptide 4 in N-CAM

Figure 14. Dephosphorylation of the sites phosphorylated on N-CAM *in vivo* inhibits phosphorylation by GSK-3 and by mouse brain extracts. Embryonic chicken N-CAM was phosphorylated and dephosphorylated as indicated, and samples were subjected to SDS-PAGE and autoradiography. N-CAM was incubated with GSK-3 and γ - 32 P-ATP (lane 1) or dephosphorylated by potato acid phosphatase and then incubated with γ - 32 P-ATP and GSK-3 (lane 2) or CK II (lane 3). N-CAM dephosphorylated by acid phosphatase was incubated with CK II and γ - 32 P-ATP then with GSK-3 and γ - 32 P-ATP (lane 4). N-CAM was incubated with the S₂ fraction of mouse brain and γ - 32 P-ATP (lane 5) or was dephosphorylated by acid phosphatase and then incubated with the S₂ fraction and γ - 32 P-ATP (lane 6). *PAP*, incubation with potato acid phosphatase; *CK II*, incubation with casein kinase II; *GSK-3*, incubation with glycogen synthase kinase-3; *S₂*, incubation with an aliquot of mouse brain S₂ fraction containing 5 μ g total protein.

immunoprecipitated from mouse reaggregate cultures has to be phosphorylated in culture on serine prior to being phosphorylated by GSK-3 on threonine (Figs. 11, 14). The observation that GSK-3 phosphorylates peptide 4/4' with a stoichiometry of approximately 1 mol/mol thus indicates that at least peptide 4 is stoichiometrically phosphorylated on serine in purified N-CAM. Phosphorylation of the *in vivo* sites *in vitro* may also be limited because the relevant kinase is present in very low concentrations in tissue extracts, is rapidly inactivated during isolation, or may require an unknown cofactor or activator. Alternatively, the secondary and higher-order structure of N-CAM may be required for phosphorylation and may be partially disrupted during solubilization and purification. Finally, N-CAM may resemble the β -adrenergic receptor (Benovic et al., 1986) in being phosphorylated *in vivo* only when it has bound the appropriate ligand. For N-CAM this might be another N-CAM molecule or a different molecule on or near the cell surface.

Purified chicken N-CAM is phosphorylated by GSK-3 or CK I on one major peptide (peptide 4') with a stoichiometry of approximately 1 mol/mol. Mouse N-CAM, phosphorylated in culture and rapidly isolated in the presence of phosphatase inhibitors, can be phosphorylated on an additional peptide (peptide 3') by GSK-3. However, the phosphorylation of peptide 3' is variable and usually less apparent than that of peptide 4'. Because the seryl residues in peptides 3 and 4 must be phosphorylated before GSK-3 can phosphorylate the threonyl residues, one explanation for the relatively low phosphorylation of peptide 3' *in vitro* is that the seryl residue is phosphorylated to a lower level *in vivo* than that in peptide 4. Similarly, peptides

3 and 4 in the sd chain are phosphorylated only to a low level in culture, consistent with the observation that the sd polypeptide of purified N-CAM is a poor substrate for GSK-3.

The amino acid sequences of the CK I phosphorylation site in casein (glu-x-thr; Hathaway and Traugh, 1982) and the GSK-3 phosphorylation sites in glycogen synthase, R_{II} and I-2 (thr-arg, thr-pro or thr-x-pro; Hemmings and Cohen, 1983; Aitken et al., 1984) are known. Assuming that the phosphorylation sites in N-CAM for GSK-3 and CK I are similar to the phosphorylation sites in the other known substrates, chicken N-CAM (Hemmerly et al., 1986a) as well as mouse and rat N-CAM (Santoni et al., 1987; Small et al., 1987), contains several potential phosphorylation sites for both enzymes. N-CAM in brain membrane vesicles is phosphorylated only after solubilization with NP-40, and the ssd polypeptide is not a substrate for GSK-3 or CK I, consistent with previous proteolytic studies (Sorkin et al., 1984) suggesting that the phosphorylation sites are intracellular. In addition, the peptides phosphorylated by GSK-3 and CK I in both the ld and sd polypeptides are similar, and are probably in a shared region of the cytoplasmic domain. Since the phosphorylation of N-CAM on seryl residues appears to regulate the extent of phosphorylation by GSK-3 or CK I, the likely phosphorylated threonyl residues should be close to seryl residues. The region from amino acid 762 in the cytoplasmic domain of either chicken N-CAM polypeptide to the carboxyl terminus is unusually rich in threonyl residues and contains a number of potential phosphorylation sites for both enzymes. The same is true in mouse and rat. However, definitive localization of the phosphorylation sites in N-CAM will require amino acid sequence of the isolated phosphopeptides.

Protein phosphorylation is a common mechanism through which diverse cellular responses are regulated, particularly in the nervous system (Greengard, 1987). Phosphorylation affects a variety of functions of many different cell surface proteins, including receptor affinity, clustering, and internalization, and interaction with the cytoskeleton or with cytoplasmic factors (Kelleher et al., 1984; May et al., 1984; Lin et al., 1986). In many cases, a single protein can be phosphorylated by different kinases, each activated by a discrete stimulus and having distinct effects on the function of the protein. We have found that N-CAM is phosphorylated by several protein kinases, giving rise to relatively stable and to transient types of phosphorylation. In addition, the sd and ld polypeptides of N-CAM are differentially phosphorylated. The phosphorylation of N-CAM on each type of site (stable, transient, and ld polypeptide-specific) may have different effects on the function of the protein. Variations in the binding activity of N-CAM play a key role at sites of induction throughout development (Edelman, 1984, 1985). Phosphorylation of the cytoplasmic domains may affect binding activity, directly or indirectly, by altering protein distribution or mobility on the cell surface or by altering interactions with other cell-surface proteins or with cytoskeletal components. Since small increases in N-CAM concentration result in large increases in N-CAM-mediated adhesion (Hoffman and Edelman, 1983), the effect of phosphorylation on any of these properties would have important histogenetic consequences.

Phosphorylation of N-CAM *in vivo* apparently occurs only when the molecule is in or close to the surface membrane (Lyles et al., 1984), suggesting that phosphorylation of these sites may be associated either with the maintenance of N-CAM at the cell membrane or with its interaction with other molecules in or near the membrane. The ld polypeptide has phosphorylation sites that are phosphorylated to different extents as well as phosphorylation sites that are distinct from those found in the sd polypeptide, suggesting that significant phosphorylation is unique to the ld polypeptide. This is of particular interest as the ld polypeptide is present only in neurons, and not in other adult tissues that express N-CAM such as skeletal muscle (Pollerberg et al., 1985; Murray et al., 1986) and has been reported to interact with brain spectrin and have a lower surface mobility than the sd polypeptide (Pollerberg et al., 1986). The differential phosphorylation of the ld polypeptide may be related to its specific interactions with molecules in the cytoskeleton or cytoplasm of neurons, while the phosphorylation of the sites that are common to the ld and sd polypeptides may regulate functions which are common to both polypeptides.

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