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Changes in neural cell adhesion molecule (NCAM) structure during vertebrate neural development

(cell–cell adhesion/polysialic acid/cytoplasmic domain/neural plasticity)

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ABSTRACT **Changes in carbohydrate and polypeptide form of the neural cell adhesion molecule (NCAM) have been documented during the development of central nervous system tissue in both chicken and frog. The carbohydrate variations reflect a high and low content of polysialic acid, and for the two vertebrates examined the expression of these forms is similar. At very early stages of neural development NCAM with a low content of polysialic acid is present, during histogenesis of the central nervous system NCAM with a high content of polysialic acid dominates, and there is a gradual return to NCAM with a low content of polysialic acid as the animals approach maturity. In contrast, the order of expression of the major NCAM polypeptide forms is different in the chicken and frog. These findings suggest that changes in sialic acid are a fundamental aspect of the function of NCAM in development, whereas NCAM polypeptide differences may affect events associated with a particular vertebrate. Studies have demonstrated that a decreased sialic acid content enhances the adhesion properties of NCAM. On this basis, we propose that NCAM with a low content of polysialic acid functions both to maintain integrity of neuroepithelium during morphogenesis of the early embryo and to stabilize differentiated structures in the adult, while the decreased adhesive function of NCAM with a high content of polysialic acid provides more plasticity in cell interactions during cell migration, axon outgrowth, and formation of neural circuits.**

The neural cell adhesion molecule (NCAM) is a cell surface glycoprotein that acts as a homophilic ligand in cell-to-cell adhesion (for review, see refs. 1 and 2). NCAM is found in the nervous system of many vertebrate species (3, 4). In the animals considered in this study, chickens and frogs, NCAM is first detected in gastrulae (ref. 5; R. W. Keane and U.R., unpublished data), continues to be expressed throughout the developing central and peripheral nervous systems (6, 7), and persists in reduced amounts in the adult (8, 9). In the later stages of neural tissue formation, NCAM is found on most if not all neurons (6, 10), glial cells (8, 11, 12), and skeletal muscle (13, 14). There is some variation among species in this pattern of expression, for example in frog, NCAM is only transiently associated with early muscle development (7). It has been proposed that NCAM-mediated adhesion among nerve, glia, and muscle has a variety of developmental roles, including axon guidance along glial cell "endfeet" (15), neurite fasciculation (10, 16–18), and the initial interaction between axons and myotubes (19).

NCAM varies in its content of α2,8-polysialic acid (PSA) (9, 20–23), from a maximum of ≈30% (wt/wt) to <10% (9, 22). These variations occur with developmental age (9, 21, 22), between different regions of a tissue (24), and even within an individual neuron (25). PSA can be specifically removed from NCAM by the soluble K1F bacteriophage enzyme endoneuraminidase (endo N), yielding glycopolypeptides of apparent M_rs 180 kDa, 140 kDa, and 120 kDa (designated NCAM-180, NCAM-140, and NCAM-120, respectively) by $NaDodSO_4$/PAGE (26, 27). Such desialylation increases the rate of NCAM-mediated cell aggregation *in vitro* (28, 29), the adherence of NCAM-bearing liposomes to neuroblastoma cells (30), and the aggregation of purified NCAM (31).

A second modulation of NCAM appears as polypeptide variants (22, 32, 33) with apparent molecular weights for the deglycosylated proteins of 160 kDa, 130 kDa, and 110 kDa by $NaDodSO_4$/PAGE. These polypeptides differ primarily in the length of the C-terminal region, which represents an intracellular domain for the larger forms (33–37). The relative amounts of the different peptides change with cell type (11, 38), degree of *in vitro* differentiation (12), developmental stage (39, 40), and region of the nervous system (21, 24).

Although these variations in NCAM form have been analyzed in a number of chicken tissues, particularly between late embryonic (day 6–10) and adult stages (9, 24, 25), a description of early developmental stages has not been reported. In this study we have used electrophoretic mobility by $NaDodSO_4$/PAGE to characterize NCAM structure in the vertebrate central nervous system (CNS) at several early embryonic stages. Comparative analyses between two phylogenetically distant species, chicken and frog, have been used to examine the significance of different NCAM forms in vertebrate development.

MATERIALS AND METHODS

Animals. The preparation of *Xenopus laevis* embryos has been described (41). Briefly, fertilized eggs were harvested following injection of human chorionic gonadotrophin into mating pairs of adult frogs. The jelly coat was removed at either the two- or four-cell stage, and the embryos were allowed to develop at room temperature in 70% (vol/vol) Steinberg's solution (5) containing Gentamicin (40 mg/liter). *Xenopus* embryos were staged according to Nieuwkoop and Faber (42). White Leghorn chicken embryos were obtained from humidified incubations of fertile eggs and were staged according to Hamburger and Hamilton (43). A local slaughterhouse provided fresh adult chicken brains.

Sample Preparation. Samples consisted of tissue homogenates, membrane vesicles or immunoaffinity purified NCAM from tissue homogenates. Whole brains were dissected from the oldest embryos (*Xenopus*, stage 54 and older; chicken, stage 30 and older); total CNS was dissected from younger embryos (*Xenopus*, stage 40; chicken, stages 20 and 25); and

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Abbreviations: CNS, central nervous system; endo N, soluble endoneuraminidase from K1F bacteriophage; NCAM, neural cell adhesion molecule; PSA, α2,8-polysialic acid; H and L, heavily and lightly sialylated material.

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the entire embryo was used from the youngest embryos (*Xenopus*, stage 28; chicken, stages 5–15). Total CNS was defined as the tissue remaining after removal of any yolk sac, endoderm, outer membranes, and limb buds. Although the entire embryo was used at very early stages, it is known that NCAM is largely confined to neural tissue in early development (ref. 7; R. W. Keane and U.R., unpublished data). All tissue (except adult chicken) was solubilized in 14 volumes of extraction buffer at 4°C by either sonication or by Dounce homogenization. The extraction buffer consisted of PBS (pH 7.4) containing 0.5% Nonidet P-40, 1 mM EDTA, and aprotinin at 100 kallikrein inhibitor units/ml. Homogenates were centrifuged for 10 min at 13,000 × *g*, and the supernatant was saved. In the chicken samples, NCAM was purified and concentrated by immunoaffinity isolation (20) using monoclonal antibody 5E, which recognizes all three major NCAM polypeptides (36, 37). For adult chicken samples, membrane vesicles were prepared prior to detergent extraction (20).

Cleavage of NCAM-Associated PSA with Endo N. Endo N was purified as described (26, 29). Tissue preparations were digested with an excess of enzyme (at least 1 unit/20 μl) for at least 1 hr at 4°C. Immunoaffinity purified NCAM was digested while still bound to the Sepharose. Enzymatic digestion was stopped by the addition of $NaDodSO_4$/PAGE sample buffer (44).

$NaDodSO_4$/PAGE Analysis. All samples were solubilized in $NaDodSO_4$/PAGE sample buffer (44) either directly from the tissue homogenates (*Xenopus*), from NCAM bound to immunoaffinity beads (embryonic chicken), or from the adult chicken vesicle suspension, and then heated to 100°C for 3 min. Different NCAM forms were detected according to their electrophoretic mobility in $NaDodSO_4$ gels (44), using a 7% separating gel with a 4% stacking gel. Sample loads were varied to create nearly equal immunoblot staining. This was not possible for the youngest stages because of low NCAM concentrations; these lanes always received the maximum possible load. Separated proteins were electrophoretically transferred from the gel to nitrocellulose (45), and the NCAM was detected by incubation with a primary polyclonal rabbit antibody: either anti-frog NCAM (5) or anti-chicken NCAM (17). The ratio of different NCAM polypeptide forms was not altered by transfer time or the use of antisera from different rabbits. Bound antibody was detected using peroxidase-conjugated goat anti-rabbit second antibody (Vector Laboratories, Burlingame, CA, or Cooper Biomedical, Malvern, PA) (46), and the enzyme reaction was developed using 0.05% 4-chloro-1-naphthol and 0.01% H_2O_2 as substrates (47). Spectrophotometric scans at 575 nm were made using a Shimadzu CS-930 dual wavelength TLC scanner. Total areas under each curve were calculated using an internal integrator. Relative areas under parts of curves were estimated from the weights of corresponding paper cutouts. Serial dilutions of a single sample produced comparable scans when normalized for staining intensity.

RESULTS

NCAM characteristically appeared on immunoblots as a diffuse, low-mobility band followed by a number of discrete bands. The diffuse staining represented heavily sialylated NCAM, and the bands corresponded to the three polypeptide forms. Endo N digestion of the PSA side chains on NCAM converted diffuse electrophoretic patterns into distinct bands that reflected each polypeptide type. Thus, a comparison of untreated and endo N-treated samples provided a qualitative assessment of the degree of sialylation in each sample.

NCAM Forms During Frog Development. Immunoblots of NCAM in *Xenopus* at selected stages from stage 28 to adult, are shown in Fig. 1. NCAM is first detectable in the neural

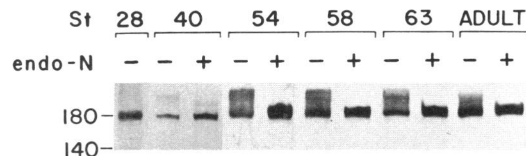

FIG. 1. NCAM forms in *Xenopus* at selected developmental stages. In these immunoblots, each stage (except stage 28) is shown without (−) and with (+) treatment of the tissue extract with endo N to cleave PSA. Stage 28 NCAM did not change mobility after endo N treatment. Molecular masses in kDa are indicated at the left. St, stage.

plate of stage 14 *Xenopus* by immunofluorescent staining of tissue sections (7). However, the molecule is not abundant enough for detection in detergent extracts of tissue by immunoblots until about stage 28 (7).

Although a polyclonal antibody that recognizes all major NCAM forms was used for the immunoblots, at stage 28 a single 180-kDa band was stained. This material did not change its mobility after endo N treatment, indicating that it contained relatively little PSA. The band was recognized in immunoblots by monoclonal antibody 4D (5), which is directed against an epitope specific for the 180-kDa form of chicken NCAM (36, 37). Thus this form of NCAM is the homologue of chicken NCAM-180 (rather than another NCAM form of identical electrophoretic mobility).

From larval stages 40 to 63, endo-N treatment resulted in large increases in electrophoretic mobility, in each case revealing the presence of a 180-kDa polypeptide. In the adult, endo N treatment only changes the mobility of a small fraction of the total NCAM, which again was the 180-kDa form. Although no NCAM-140 was found in our immunoblots of early embryos, it has been reported that the ratio of different NCAM transcripts remains constant throughout development (48). When large amounts of material were analyzed, NCAM-140 was detected at low levels in immunoblots of stage 55 frogs (data not shown), and in adults (Fig. 2). It is, therefore, likely that smaller polypeptide forms of NCAM are present in small amounts throughout development.

Because the developmental expression of NCAM polypeptide forms in frog is markedly different than in chicken (see below), additional tests were carried out to validate the methods used to detect them. Although the relative abundance of NCAM-180 was not due to a restricted specificity of the polyclonal antibody used in the immunoblots (Fig. 2), the

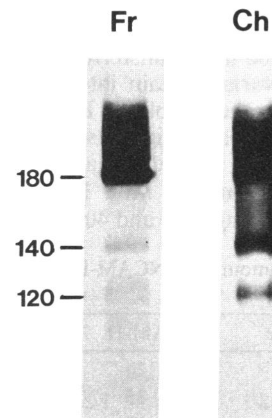

FIG. 2. NCAM forms in immunoblots as detected by polyclonal antisera. Ch, stage 45 chicken brain with rabbit anti-chicken NCAM. Fr, adult frog brain with rabbit anti-frog NCAM. The absence of 140-kDa and 120-kDa material in Fig. 1 reflects the dominance of frog NCAM-180 shown here. Molecular masses are labeled in kDa.

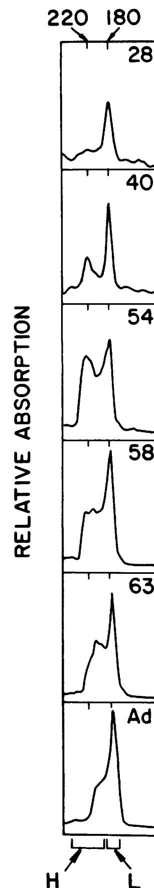

FIG. 3. Reflectance scans of NCAM forms in *Xenopus*. Untreated (−) lanes from Fig. 1 were scanned; the areas and positions were normalized and aligned with respect to the 180-kDa peak, as marked. Brackets labeled L and H represent areas with low and high levels of NCAM sialylation. Ad, adult.

possibilities remained that a particular antibody preferentially bound NCAM-180 or that the transfer and immunoblot procedures detected NCAM-180 more efficiently than NCAM-140. However, similar results were obtained with a variety of antibodies that react with frog-NCAM, including monoclonal antibodies directed against carbohydrate epitopes that appear to be shared by each polypeptide variant (36, 37). Large differences in the transfer efficiencies of different NCAM forms appear unlikely in that immunoprecipitated adult frog NCAM also displayed a relative abundance of NCAM-180 in untransferred, silver-stained gels.

To correct for variable stain intensities and to quantify levels of sialylation, the samples in Fig. 1 were scanned (Fig. 3). These normalized and aligned scans revealed two components: H, a heavily sialylated material, and L, a 180-kDa polypeptide with relatively little PSA. Most of the frog NCAM detected at stages 28 and 40 and in adults was found in the L region (hereafter termed NCAM-L), while the late larval stages 54, 58, and 63 had a more equal distribution of NCAM-L and NCAM-H (Fig. 3 and Table 1). The extent of sialylation reached a maximum about stage 54 (Table 1).

Table 1. Relative amounts of NCAM-H and NCAM-L during frog development

Xenopus stage	NCAM-H, %	NCAM-L, %
28	20	80
40	35	65
54	60	40
58	45	55
63	45	55
Adult	30	70

Percentages represent areas in bracketed sections of the scans in Fig. 3.

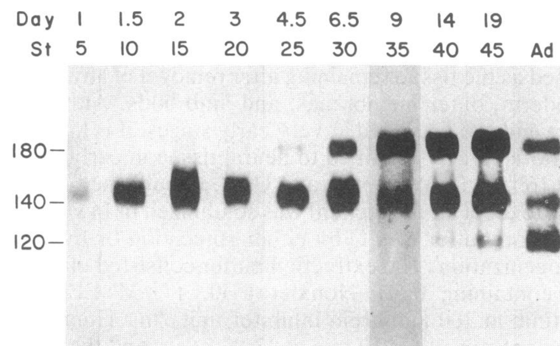

FIG. 4. NCAM polypeptides in chicken at selected developmental stages. These immunoblots revealed NCAM after exposure to endo N. Molecular masses in kDa are indicated. Ad, adult.

NCAM Forms During Chicken Development. As reported, chicken brain contains NCAM-180 (9), NCAM-140 (20), and NCAM-120 (21, 22). The changes in expression of these forms during development, as revealed by immunoblots after treatment with endo N, are shown in Fig. 4. The first polypeptide detected in the chicken was NCAM-140 (stage 5). This polypeptide form predominated in the chicken through stage 15 (50–55 hr). Only later, by stage 20 (day 3), did both NCAM-180 and NCAM-140 appear. Thereafter, chicken NCAM-180 staining intensified until stage 35 (day 9), when it equaled or exceeded that of NCAM-140. The third polypeptide form NCAM-120 was first detectable at approximately stage 40 (day 14) and increased to a maximum in the adult.

The variation in PSA content of NCAM in the chicken CNS followed a similar pattern to frog (Fig. 5). That is, NCAM-L predominated in the early embryo, then NCAM-H increased to a maximum at late embryonic stages, and finally NCAM-L reappeared in the adult. The earliest chicken stage examined (stage 5, definitive streak) expressed mostly NCAM-L, as did the adult. NCAM-H appeared in stage 10 embryos (10 somites), increased in amount at stage 15, and remained at high levels through stage 45. The shift between stage 25 and stage 30 represents the added expression of NCAM-180 as depicted in Fig. 4, not an increase in the PSA content of NCAM-140.

When the lanes in Fig. 5 were scanned, two regions could again be identified: the NCAM-L forms, which appeared as peaks with molecular weights of 180 kDa, 140 kDa, and 120 kDa and the NCAM-H forms to their left (Fig. 6). There was a clear shift from NCAM-L at stage 5 to NCAM-H by stage

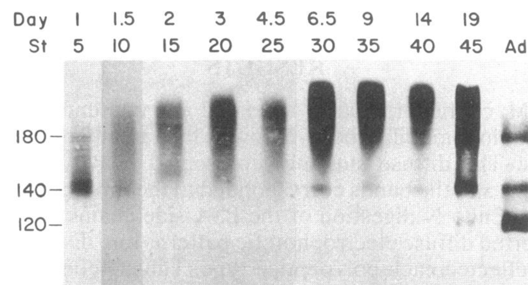

FIG. 5. NCAM in chicken at selected developmental stages. NCAM was detected in immunoblots of the same samples as in Fig. 4, but without exposure to enzyme. Molecular masses in kDa are indicated. St, stage; Ad, adult.

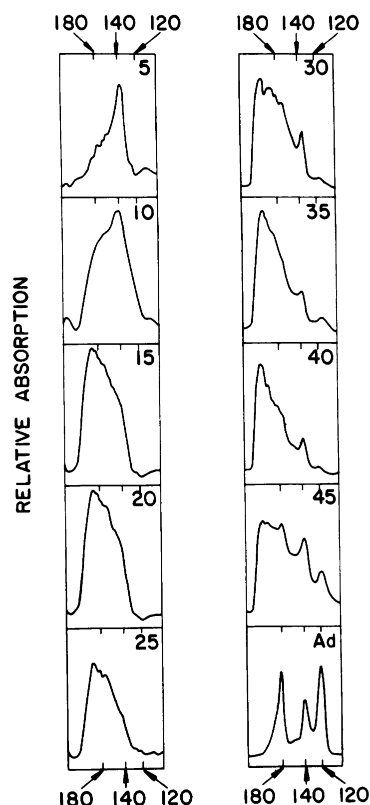

FIG. 6. Reflectance scans of NCAM forms in chicken. The lanes shown in Fig. 5 were scanned; the areas were normalized with respect to maximum reflectance, and the scans were aligned by the 120-kDa, 140-kDa, and 180-kDa NCAM peaks, as marked. Any material not within the area of these peaks represents sialylated NCAM. Ad, adult.

15. Simultaneous expression of more than one polypeptide form in the chicken precluded an unambiguous separation and quantification by $NaDodSO_4$/PAGE of NCAM-H and -L. Nevertheless, if all material present in the 180-kDa, 140-kDa, and 120-kDa peaks represented NCAM-L rather than NCAM-H, then integration of the scans still demonstrated a progression from 40% NCAM-H to 60% NCAM-L at stage 5, to a maximum of 70% NCAM-H to 30% NCAM-L in stages 15–40, and finally 20% NCAM-H to 80% NCAM-L in the adult.

DISCUSSION

These studies document marked changes in the structure of NCAM over the course of vertebrate CNS development. The changes involve alterations in the PSA content of the molecule and the length of its polypeptide chain. In a comparison between frog and chicken, the patterns of changes in NCAM sialylation were found to be similar, with a low PSA content (L form) during early embryonic stages, a dramatic increase to the highest PSA content (H form) during later embryonic development, followed by a gradual return to an L form in the adult. In contrast, the variations of polypeptide form differed between these species. With chicken, NCAM-140 was expressed first, joined later by substantial amounts of NCAM-180 and NCAM-120. With frog, NCAM-180 appeared to be the dominant form throughout development and in the adult.

This surprisingly different pattern of expression of NCAM-140 and NCAM-180 between frog and chicken suggests that either some aspects of NCAM function are relatively insensitive to changes in the length of the polypeptide, or these two vertebrates have important differences in their use of NCAM during development. The major feature that distinguishes NCAM-180 from NCAM-140 is the size of the intracellular domain. Assuming that intracellular differences are more likely to reflect functions that regulate or are triggered by cell adhesion, this difference could reflect the control or consequences of adhesion rather than a change in intracellular binding.

The presence of similar patterns of variation in the PSA content of NCAM during frog and chicken development, independent of polypeptide form, suggests that these carbohydrate changes are associated with fundamental aspects of vertebrate CNS formation. This premise is consistent with previous reports on changes in NCAM carbohydrate. An analysis of PSA on NCAM in the developing chick retinotectal projection suggests that NCAM carbohydrate content reflects changing needs for optic axon fasciculation (25), NCAM-L is proposed to have a role in controlling granule cell migration in the cerebellum (32), and NCAM sialic acid levels in selected regions of the CNS are correlated with synaptogenesis (24). In some of these studies, the change in PSA on NCAM is interpreted in terms of a shift from "embryonic" to "adult" forms of NCAM (9, 22). However, our present work has revealed that an "adult-like" form also exists in the early embryo, and it is likely that detailed analyses of individual tissues will reveal additional variations in PSA. Therefore, it would appear that NCAM carbohydrate forms are more effectively named according to their structural properties (H and L) rather than a developmental stage.

In view of the inverse relationship between PSA content and the rate of NCAM-mediated adhesion *in vitro* (28–31), a simple interpretation of these results would be that NCAM-L is produced at times when neural tissue formation requires enhanced cell–cell binding, and that NCAM-H is expressed whenever less NCAM-mediated cell adhesion is necessary. On this basis we propose that the observed regulation of PSA content provides strength in cell–cell bonding during initial morphogenesis, plasticity in cell interactions during histogenesis, and finally a stabilization of contacts and positions in mature tissues. For example, in early stages of development, the neuroepithelium must be able to withstand mechanical stresses associated with formation of the neural tube, flexures, and evaginations. Subsequently, however, differentiating neurons need more freedom to migrate, extend neurites, and innervate appropriate targets. Such events require more selective recognition mechanisms whose effectiveness may be enhanced by a decreased level of general adhesiveness. Finally, we view the return of L forms in the adult as a means of stabilizing the position and connections of fully differentiated neurons.

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