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SITE-DIRECTED ANTIBODIES TO LOW-VOLTAGE-ACTIVATED CALCIUM CHANNEL $Ca_V3.3$ (ALPHA1I) SUBUNIT ALSO TARGET NEURAL CELL ADHESION MOLECULE-180

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Abstract—Synthetic peptides of defined amino acid sequence are commonly used as unique antigens for production of antibodies to more complex target proteins. We previously showed that an affinity-purified, site-directed polyclonal antibody (CW90) raised against a peptide antigen (CNGRMPNIAKDVFTKM) anticipated to be specific to a T-type voltage-dependent Ca^{2+} channel subunit identified recombinant rat alpha1I/$Ca_V3.3$ and two endogenous mouse proteins distinct in their developmental expression and apparent molecular mass (neonatal form 260 kDa, mature form 190 kDa) [Yunker AM, Sharp AH, Sundarraj S, Ranganathan V, Copeland TD, McEnery MW (2003) Immunological characterization of T-type voltage-dependent calcium channel $Ca_V3.1$ (alpha 1G) and $Ca_V3.3$ (alpha 1I) isoforms reveal differences in their localization, expression, and neural development. Neuroscience 117:321–335]. In the present study, we further characterize the biochemical properties of the CW90 antigens. We show for the first time that recombinant alpha1I/$Ca_V3.3$ is modified by *N*-glycosylation. Using peptide:*N*-glycosidase F (PNGase F), an enzyme that removes polysaccharides attached at Asn residues, and endoneuraminidase-N (Endo-N), which specifically removes polysialic acid modifications, we reveal that differential glycosylation fully accounts for the large difference in apparent molecular mass between neonatal and adult CW90 antigens and that the neonatal form is polysialylated. As very few proteins are substrates for Endo-N, we carried out extensive analyses and herein present evidence that CW90 reacts with recombinant alpha1I/$Ca_V3.3$ as well as endogenous neural cell adhesion molecule-180 (NCAM-180). We demonstrate the basis for CW90 cross-reactivity is a five amino acid epitope (AKDVF) present in both alpha1I/$Ca_V3.3$ and NCAM-180. To extend these findings, we introduce a novel polyclonal anti-peptide antibody (CW678) that uniquely recognizes NCAM-180 and a new antibody (CW109) against alpha1I/$Ca_V3.3$. Western blot analyses obtained with CW678, CW109 and CW90 on a variety of samples confirm that the endogenous CW90 signals are fully attributed to the two developmental forms of NCAM-180. Using CW678, we present novel data on differentiation-dependent NCAM-180 expression in human neuroblastoma IMR32 cells. These results strongly suggest the need for careful analyses to validate anti-peptide antibodies when targeting membrane proteins of low abundance.

Key words: *N*-linked glycosylation, human neuroblastoma IMR32 cells, T-type, differentiation, development, polysialylation.

Site-directed, anti-peptide antibodies remain useful tools in the identification of recombinantly expressed proteins and endogenous antigens (McEnery et al., 1997; Sharp et al., 1995, 2001; Westenbroek et al., 1990; Sakurai et al., 1996). The wealth of primary sequence data currently available to investigators has led to a surge in the production of anti-peptide antibodies that demonstrate unique specificity for the target protein under a variety of experimental conditions. Key to the production of anti-peptide antibodies is the choice of peptide antigen (Angeletti, 1999). The procedure involves several parallel considerations including: 1) searching for short segments of primary sequences likely to be unique to the protein isoform of interest by carrying out BLAST searches among related family members followed by extensive screening of several databases at different stringencies, 2) evaluating the peptide for its hydrophilic quality both as a chemically synthesized product and in its native conformation, 3) predicting the accessibility of the chosen segment of primary sequence to antibodies, 4) evaluating the predicted secondary structure of the peptide, 5) estimating the ease of chemical synthesis of the peptide and its coupling to a carrier protein, 6) having access to a non-ambiguous positive control for the protein of interest expressed in a heterologous cell line that does not express endogenous pro-

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Abbreviations: BrdU, 5-bromo-2-deoxyuridine; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; EMEM, Eagle's minimal essential medium; Endo-N, endoneuraminidase-N; GlcNAc, *N*-acetylglucosamine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); HVA, high-voltage activated; KLH, keyhole limpet hemocyanin; LVA, low-voltage activated; MAP, microtubule-associated protein; NCAM-180, neural cell adhesion molecule-180; Neo, neonatal; P, postnatal day; PMSF, phenylmethanesulfonyl fluoride; PNGase F, peptide:*N*-glycosidase F; PSA, polysialic acid; TBS, Tris-buffered saline; VDCC, voltage-dependent Ca^{2+} channels; VGSC, voltage-gated sodium channel; WGA, wheat germ agglutinin.

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tein, and 7) having access to a negative control sample, such as an engineered knock-out animal.

The utilization of anti-peptide antibodies has great value for discriminating among closely related members of a specific protein family, such as the family of proteins that comprise the large class of neuronal voltage-dependent calcium channels (VDCC) (Catterall 2000; Perez-Reyes 2003). VDCC are composed of two structurally and functionally distinct channel families classified as high-voltage activated VDCC (HVA) and low-voltage activated (LVA). While HVAs conduct large and long-lasting currents that control neurotransmitter release, gene expression, neurite extension, and intracellular signaling (Catterall, 2000; Perez-Reyes, 2003), LVAs may gate the activity of other voltage-activated ion channels (Llinas and Jahnsen, 1982), resulting in burst firing and intrinsic membrane potential oscillations (Huguenard, 1996; McCormick and Bal, 1997). The design, characterization, and application of panels of specific, anti-peptide antibodies have added greatly to our understanding of many biological and physiological properties of VDCC including their subcellular localization (Westenbroek et al., 1995; Jones et al., 1997; Sakurai et al., 1996), subunit assembly (Vance et al., 1998; McEnery et al., 1998a; Pichler et al., 1997), and developmental pattern of expression (Jones et al., 1997; Vance et al., 1998; McEnery et al., 1998b).

With the goal of better understanding the developmental expression and biochemical properties of the individual members of the LVA VDCC family of channel proteins, we focused on producing site-directed, anti-peptide antibodies that were designed to distinguish among the three alpha1 subunits (termed alpha1G/Ca_V3.1, alpha1H/Ca_V3.2, and alpha1I/Ca_V3.3) that define the three LVA VDCC gene families. To generate site-directed antibodies to alpha1I/Ca_V3.3 we followed the above strategies for epitope selection and identified a sequence (CNGRMPNIAKDVFTKM) predicted to be present in the mouse (accession number XP_991079, corresponding to amino acids 1168–1183) and rat (accession number AF086827, corresponding to amino acids 1053–1068) alpha1I/Ca_V3.3 subunit. This sequence was chosen because it was unique to alpha1I/Ca_V3.3 when compared with homologous proteins Cav3.1 (accession number NM_031601) and Ca_V3.2 (accession number NM_153814) (Fig. 1). Furthermore, this sequence offered the opportunity to utilize an endogenous cysteine residue for coupling the peptide to either keyhole limpet hemocyanin (KLH) carrier protein or a Sulfolink column. We produced an anti-peptide antibody (CW90) and reported the specificity of CW90 toward recombinant rat alpha1I/Ca_V3.3 (accession number: AF086827) expressed in HEK293 cells, with no signal evident in non-transfected cells. We next applied CW90 to the identification of alpha1I/Ca_V3.3 in endogenous samples, and detected two endogenous antigens; a neonatal (Neo) form of the antigen (260 kDa) and an adult form (190 kDa). All signals detected were eliminated by the preincubation of CW90 with excess antigenic peptide (Yunker et al., 2003).

It was clear from the outset that the pattern of immunoreactivity observed for the CW90 antigens, i.e. a developmental change in apparent molecular mass between postnatal days (P) 0 and 15, had not been previously reported for a VDCC (Yunker et al., 2003). However, the large range in the apparent molecular mass of the CW90 antigens was consistent with the diversity in size reported for alpha1I/Ca_V3.3 splice variants that had been previously published (Chemin et al., 2001; Mittman et al., 1999; Perez-Reyes 2003; Murbartian et al., 2004). Also, LVA VDCC alpha1 subunits are structurally homologous to the pore-forming alpha subunit of another family of voltage-gated ion channels (voltage-gated sodium channels, VGSC) (Tsien, 1998; Jones, 2003). VGSC alpha subunits undergo developmental maturation by way of post-translational modification (Tyrrell et al., 2001; Schmidt and Catterall, 1986; Castillo et al., 1997). Lastly, there was the circumstantial evidence that suggested a relationship between the decrease in the apparent molecular mass of the CW90 antigens and the increase in molecular mass of Ca_V3.1 as detected by CW53, another site-directed antibody previously validated using a Ca_V3.1 knock-out mouse (Kim et al., 2001). With these observations, we hypothesized several mechanisms that could potentially contribute to the large difference in the apparent molecular mass we observed in the CW90 antigens during development (Yunker et al., 2003).

As the present study reports, despite adhering to the strategies for anti-peptide antibody production that had been successfully applied in the past, and despite rigorous efforts to evaluate the specificity of CW90 for its predicted antigen, alpha1I/Ca_V3.3, the outcome of this undertaking was very different than anticipated. As we initiated the side-by-side comparison of the effects of deglycosidase treatment of both recombinantly expressed alpha1I/Ca_V3.3 and the endogenous CW90 antigens to determine if they were differentially glycosylated in development, we noted properties that clearly distinguished among the recombinant and native samples that were not easily reconciled. We therefore undertook a careful analysis of the biochemical properties of the endogenous CW90 antigen.

Indeed, as first reported, CW90 identifies heterologously expressed alpha1I/Ca_V3.3 in HEK293 cells, but herein we report the unexpected finding that CW90 also robustly and exclusively identifies neural cell adhesion molecule-180 (NCAM-180) in native samples. This finding was quite surprising as sequence similarity between

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Cav3.3  AF086827  1045 QAPGHEDCNGRMPNIAKDVFTKMD-DRRDRGEDEEE 1108
Cav3.2  NM_153814 1220 ---KFHDCNGQMVALPSEFFLRIDSHKEDAAEFDDD 1284
Cav3.1  NM_031601 1170 SASEHQDCNGKSASGRLARTLRTDDPQLDGDDDNDE 1234
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Fig. 1. Alignment of alpha1I/Ca_V3.3 with Ca_V3.2 and Ca_V3.1 identifies CW90 peptide as unique to alpha1I/Ca_V3.3 protein. Using the multiple alignment of protein sequences program, ClustalW, a portion of the rat Ca_V3.1, Ca_V3.2 and alpha1I/Ca_V3.3 channels is compared. The location of the CW90 peptide is underlined in the alpha1I/Ca_V3.3 sequence. The residues are colored according to the structural properties of the individual amino acid: black, positively charged; celadon, negatively charged; blue, polar; amber, non-polar.