Association of the HNK-1 epitope with 5'-nucleotidase from *Torpedo marmorata* (electric ray) electric organ

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5'-Nucleotidase isolated from the electric organ of the electric ray (*Torpedo marmorata*) has a molecular mass of 62 kDa and, on two-dimensional electrophoresis, separates into up to 13 isoforms within a pI range of 5.9–6.7. The N-terminal sequence data show a 71 % identity over 17 amino acids with that previously published for the rat liver enzyme. All forms of 5'-nucleotidase are recognized by the HNK-1 monoclonal antibody. HNK-1 immunoreactivity is found at the surface of the Schwann-cell processes covering the synaptic terminals and in this respect corresponds to that of 5'-nucleotidase in the same tissue. Since a number of glycoproteins involved in cell recognition and cell adhesion carry the HNK-1 epitope, 5'-nucleotidase may play a role in cell–cell or cell–extracellular matrix interaction in addition to its activity as an enzyme.

INTRODUCTION

5'-Nucleotidase (EC 3.1.3.5) has been identified as the enzyme responsible for the dephosphorylation of extracellular mononucleotides in a large variety of cellular systems. The primary structure of rat liver 5'-nucleotidase deduced from cDNA reveals five potential N-linked glycosylation sites and a highly hydrophobic amino acid sequence at the C-terminus as a possible signal for post-translational modification by glycosylphospholipid. The molecular mass calculated from the cDNA sequence amounts to 63 965 Da (Misumi *et al.*, 1990). The enzyme is linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) lipid anchor, as demonstrated by the equimolar presence of myo-inositol in the isolated enzyme (Baillyes *et al.*, 1990) and its release from the membrane by treatment with phosphatidylinositol-specific phospholipase C (Shukla *et al.*, 1980; Grondal & Zimmermann, 1987; Thompson *et al.*, 1987; Stochaj *et al.*, 1989a; Misumi *et al.*, 1990). It shares this property with a number of other ectoenzymes, such as alkaline phosphatase, acetylcholinesterase or various peptidases, but also with proteins involved in cell adhesion, like the neuronal cell-adhesion molecule N-CAM of 120 kDa, heparan sulphate proteoglycan or the lymphocyte-derived membrane proteins Thy-1 or LFA-3 (Hooper & Turner, 1988; Low, 1988). In addition to its properties as an enzyme, 5'-nucleotidase may be involved in cell–extracellular-matrix interactions. It has been suggested that chicken gizzard 5'-nucleotidase binds to laminin and fibronectin (Stochaj *et al.*, 1989b) and that it acts as a receptor for fibronectin in a manner different from the interaction of fibronectin with integrins (Stochaj *et al.*, 1990).

An antigenic determinant common to a number of cell-surface glycoconjugates is HNK-1. The epitope recognized by the HNK-1 antibody in glycolipids has been identified as glucuronic acid 3-sulphate (Chou *et al.*, 1986; Ariga *et al.*, 1987). The same or a very similar structure is presumably responsible for HNK-1 reactivity of glycoprotein and proteoglycan oligosaccharides. The latter include proteins involved in cell adhesion such as N-CAM, L1, J1, cytotoxic or the myelin protein MAG (Künemund *et al.*,

1988). Interestingly, a subset of acetylcholinesterase (amphiphilic forms, but not the collagen-tailed asymmetric forms) from the electric organs of the electric ray (*Torpedo marmorata*) and electric eel (*Electrophorus electricus*) are recognized by a HNK-1 antibody (Mailly *et al.*, 1989). This enzyme is similar to 5'-nucleotidase in molecular mass. It also shares its glycoprotein nature, the GPI anchor and its lectin-binding properties (Méflah *et al.*, 1984).

We have previously purified and characterized 5'-nucleotidase from the electric organ of the electric ray (Grondal & Zimmermann, 1987; Grondal *et al.*, 1988). We report here that this 5'-nucleotidase binds the HNK-1 antibody after one- and two-dimensional gel electrophoresis. The protein characterized is clearly different from acetylcholinesterase, and its N-terminal protein sequence corresponds closely to that reported for the rat liver enzyme. The HNK-1 antibody recognizes the terminal network of axons in the electric organ, binds to the surface of the Schwann-cell processes surrounding the axon terminals and thus is similar to the cellular distribution of 5'-nucleotidase.

EXPERIMENTAL

Enzyme purification

5'-Nucleotidase was purified from the electric organ of the electric ray (*Torpedo marmorata*) essentially as described by Grondal & Zimmermann (1987). This involved differential solubilization with Triton X-100 and deoxycholate, and affinity chromatography using concanavalin A-Sepharose and AMP-Sepharose (both from Pharmacia).

Electrophoretical techniques and immunodetection

PAGE (Laemmli, 1970) was carried out on minigels (thickness 1 mm) with acrylamide concentrations of 5 % (w/v) for the stacking gel and 10 % (w/v) for the running gel. Two-dimensional PAGE was performed according to O'Farrell (1975) in the presence of a carbamylated creatine phosphokinase standard (Pharmacia). Proteins were identified by silver staining (Koepsell *et al.*, 1984), and immunodetection was performed using iodine-

Abbreviation used: GPI, glycosylphosphatidylinositol.

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ated or horseradish peroxidase-conjugated second antibody (Volkandt *et al.*, 1987) after transfer to nitrocellulose (Towbin *et al.*, 1979). 5'-Nucleotidase and the HNK-1 epitope were detected using a polyclonal antibody (Grondal & Zimmermann, 1987) and a monoclonal antibody (IGM; prepared from hybridomas, A.T.C.C. TIB 200, obtained from the American Type Culture Collection, Rockville, MD, U.S.A.) respectively. In control experiments only the second antibody was applied.

Analytical procedures

Activities of 5'-nucleotidase and acetylcholinesterase (EC 3.1.1.7) were determined by the methods of Lanzetta *et al.* (1979) and Ellman *et al.* (1961) respectively. Protein concentrations were measured as described by Peterson (1977), with BSA (Sigma) as a standard.

Determination of the N-terminal amino acid sequence of 5'-nucleotidase

Approx. 50 µg of the enzyme purified by AMP-Sepharose chromatography was subjected to SDS/PAGE. The band containing 5'-nucleotidase was cut out from the gel and electroeluted. After extensive dialysis (5 mM-Tris buffer, pH, 7.4, followed by water) the sample was freeze-dried. Subsequent sequence analysis was carried out on a gas-phase protein sequencer (Applied Biosystems 470 A). Amino acids were identified by on line h.p.l.c. (RPC 18; Applied Biosystems 120A). The sequence analysis data of the *Torpedo* electric-organ 5'-nucleotidase were compared with those from rat liver using the FASTP program (Lipman & Pearson, 1985) on a VAX 8530 computer.

Immunocytochemical techniques

Tissue fixation, processing of samples and immunolabelling with peroxidase were performed essentially as described previously (Janetzko *et al.*, 1989).

RESULTS

The isolation procedure used (Grondal & Zimmermann, 1987) results in the solubilization of GPI-anchored proteins and thus releases both acetylcholinesterase and 5'-nucleotidase from the plasma membrane. After concanavalin A-Sepharose chromatography, a variety of bands, including proteins of molecular masses of 62 kDa and 66 kDa, were detected by the HNK-1 antibody (Fig. 1, a). The 62 kDa and 66 kDa components could be separated during the subsequent purification step on AMP-Sepharose. The 66 kDa protein did not bind to the column material, and was readily separated from the 62 kDa protein, which, after binding to the column, could be eluted using 10 mM-AMP as eluent (Fig. 1, b and c). Since activities of both acetylcholinesterase and 5'-nucleotidase were present in the effluent of the concanavalin A-Sepharose column, but could be separated by AMP-Sepharose (Fig. 1), it is likely that the 66 kDa and 62 kDa components represented acetylcholinesterase and 5'-nucleotidase respectively. This is further supported by the observation that a monospecific antibody directed against *Torpedo* electric-organ 5'-nucleotidase recognized only the 62 kDa protein irrespectively of whether the 66 kDa protein was present or not (Fig. 1, e-g). This also shows that our polyclonal anti-5'-nucleotidase antibody does not recognize the HNK-1 epitope.

When the 62 kDa protein was electroeluted after AMP-Sepharose chromatography and SDS/PAGE, the N-terminal amino acid sequence could be determined (Fig. 2). The results demonstrate 71% identity, over 17 amino acids, with that deduced from the nucleotide sequence of the rat liver enzyme, and an additional 18% of the amino acids represent conservative substitutions.

On two-dimensional gel electrophoresis, 5'-nucleotidase reveals up to 13 isoforms within a broad pI range of 5.9-6.7 (Fig. 3). The entire range of isoforms is recognized by both the HNK-1 antibody and the antibody to 5'-nucleotidase.

By using indirect immunocytochemistry with the HNK-1 antibody and the PAP technique, the intense arborizations of the axons at the ventral surface of the electroplaque cells, as well as the synapse-forming axon terminals, could clearly be depicted (Fig. 4a). At the electron-microscopic level (Fig. 4b), antibody

Fig. 1. Binding of the HNK-1 antibody to 5'-nucleotidase from *Torpedo* electric organ

a-c, Western blot using the monoclonal HNK-1 antibody and horseradish peroxidase-conjugated second antibody. a, Proteins eluted from concanavalin A-Sepharose. b, Proteins that did not bind to AMP-Sepharose. c, Protein (5'-nucleotidase) eluted from the AMP-Sepharose column. d, Silver staining of protein (5'-nucleotidase) after chromatography on AMP-Sepharose. e-g, Western blots using a monospecific polyclonal antibody against *Torpedo* electric-organ 5'-nucleotidase and an iodinated second antibody. e-g correspond to a-c. Protein loaded per lane corresponds to 5-10 µg for a, b, e and f, and to 0.5 µg for c, d and g. Enzyme activities of samples loaded were 71.7 (a), 69.8 (b) and 0 (c) µmol/min per mg of protein for acetylcholinesterase and 89.8 (a), 0 (b) and 2407 (c) nmol of P_i/min per mg of protein for 5'-nucleotidase.

Fig. 2. Comparison of the N-terminal sequence of 5'-nucleotidase from *Torpedo marmorata* electric organ (TEO), as determined by sequence analysis, with that of rat liver (RL) as deduced from the nucleotide sequence by Misumi *et al.* (1990)

O, Identity (I); ◆, conservative substitution (CS).

Fig. 3. Analysis of 5'-nucleotidase by two-dimensional electrophoresis

a, Silver staining of protein; b, Western blot using anti-5'-nucleotidase antibody; c, Western blot using HNK-1 antibody; d, carbamylated creatine phosphokinase standard.

Fig. 4. Immunocytochemical localization (immunoperoxidase) of HNK-1-binding sites in the *Torpedo* electric organ

(a) Network of terminal-axon branching at the ventral surface of an electroplaque cell (*en face* view). (b) Localization of reaction product at the surface of the Schwann-cell processes (arrows) covering the synaptic axon terminals (n). Similarly the Schwann-cell sheath around a preterminal axon segment (ax) is labelled. Note the intense reaction product associated with basal lamina surrounding Schwann cells and covering the surface of the postsynaptic electroplaque cell (el). Further abbreviations: is, interstitial space; ps, postsynaptic membrane. Bars correspond to 10 μ m in (a) and 1 μ m in (b).

binding could be attributed to the surface of the Schwann-cell processes ensheathing the terminal axon segments. Although diffusion of the reaction product may occur, antibody binding appears to be extended into the basal lamina covering both the electroplaque cells and the Schwann cells. When the first antibody was omitted from the incubation protocol, no significant staining could be observed by either optical or electron microscopy (results not shown).

DISCUSSION

5'-Nucleotidase is translated on membrane-bound polysomes as a lower-molecular-mass precursor that is subjected to cleavage of the signal peptide and core glycosylation. It is subsequently processed in the Golgi complex to its final form, which, in many mammalian tissues, comprises a 72 kDa glycoprotein (van den Bosch *et al.*, 1986; Wada *et al.*, 1986; Baron & Luzio, 1987). The mature enzyme has been reported to carry four oligosaccharide side chains (three of the complex type and one of the high-mannose type) (human chorionic cells; Burgemeister *et al.*, 1990). In accordance with our results on the *Torpedo* electric organ, 5'-nucleotidase from the placenta could be resolved into up to 13 isoforms spanning a pI range of 5.8–7.0 (Buschette-Brambrink & Gutensohn, 1989). Since digestion with neuraminidase (from *Clostridium perfringens*) reduces the number of spots towards alkaline forms, the differences in pI have been related to different contents of sialic acid residues in the isoforms. Although the apparent molecular mass of the *Torpedo* 5'-nucleotidase is only 62 kDa, the close similarity of the N-terminal-amino-acid sequence to that of the rat liver enzyme (Misumi *et al.*, 1990) suggests that the protein structure is largely conserved during evolution.

As an increasing number of proteins are discovered to carry the HNK-1 epitope, its functional implication becomes less than clear. It is, however, obvious that HNK-1 is associated with a number of proteins involved in cell adhesion or cell recognition, and thus it may play an important role in proliferation, differentiation, and other developmental events in the nervous system and also other tissues. Accordingly, in the developing brain, the HNK-1 epitope is maximally expressed during late embryonic life and declines during postnatal life (Prasadara

et al., 1990, and references cited therein). Surprisingly, acetylcholinesterase (Mailly *et al.*, 1989) and 5'-nucleotidase appear to be recognized by HNK-1 only in electric-fish electric organs, but not in the tissues of other species; our preliminary results suggest that 5'-nucleotidase isolated from either bovine adrenal medulla or cerebral cortex (and recognized by monospecific anti-5'-nucleotidase antibodies) do not bind the HNK-1 antibody. This would be in accordance with the observation of Mailly *et al.* (1989) that acetylcholinesterase from species other than electric ray and electric eel is HNK-1-negative. Since the electrocytes and thus the electric organs grow during the lifetime of the fish, the nerve terminals in these tissues will keep sprouting continuously and thus proteins involved in cellular interaction may remain in an 'embryonic' state.

The total cellular distribution of the HNK-1 epitope in the electric organ reflects that of a variety of glycoproteins (including acetylcholinesterase and 5'-nucleotidase) and glycolipids (Borroni *et al.*, 1989). In spite of this, the pattern of antibody binding revealed by immunocytochemistry corresponds closely to that previously observed for 5'-nucleotidase (Grondal & Zimmermann, 1987; Grondal *et al.*, 1988). 5'-Nucleotidase is associated with Schwann-cell surfaces, but also with the myelin and with the perineural sheaths. At present it is not known why extracellular AMP-hydrolysing enzyme activity should be associated with all of these sites.

The financial support of the Deutsche Forschungsgemeinschaft (SFB 169, A 10) and the U.S. National Institutes of Health (NS-26283) is gratefully acknowledged. We thank Dr. Walter Volkandt for helpful advice, and Andrea Winter for excellent technical support.

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Received 21 January 1991/1 March 1991; accepted 6 March 1991