The Adhesion Molecule on Glia (AMOG) Is a Homologue of the β Subunit of the Na,K-ATPase

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Abstract. AMOG (adhesion molecule on glia) is a Ca²⁺-independent adhesion molecule which mediates selective neuron–astrocyte interaction in vitro (Antonicek et al., 1987. J. Cell Biol. 104:1587–1595). Here we report the structure of AMOG and its association with the Na,K-ATPase. The complete cDNA sequence of mouse AMOG revealed 40% amino acid identity with the previously cloned subunit of rat brain Na,K-ATPase. Immunoaffinity-purified AMOG and the β subunit of detergent-purified brain Na,K-ATPase had identical apparent molecular weights, and were immunologically cross-reactive. Immunoaffinity-purified AMOG was associated with a protein of 100,000 Mr. Monoclonal antibodies revealed that this associated protein comprised the α2 (and possibly α3) isoforms of the Na,K-ATPase catalytic subunit, but not α1. The monoclonal AMOG antibody that blocks adhesion was shown to interact with Na,K-ATPase in intact cultured astrocytes by its ability to increase ouabain-inhibitable Sr²⁺ uptake. AMOG-mediated adhesion occurred, however, both at 4°C and in the presence of ouabain, an inhibitor of the Na,K-ATPase. Both AMOG and the β subunit are predicted to be extracellularly exposed glycoproteins with single transmembrane segments, quite different in structure from the Na,K-ATPase α subunit or any other ion pump. We hypothesize that AMOG or variants of the β subunit of the Na,K-ATPase, tightly associated with an α subunit, are recognition elements for adhesion that subsequently link cell adhesion with ion transport.

The Ca²⁺-independent adhesion molecule on glia (AMOG) has been implicated in developmental events in the nervous system. It is expressed by central nervous system glial cells and is involved in neuron–astrocyte, but not astrocyte–astrocyte adhesion in vitro (Antonicek et al., 1987). In tissue sections, AMOG is detectable in the early postnatal cerebellum on Bergmann glial cells during granule cell migration. Fab fragments of monoclonal AMOG antibodies strongly inhibit this migration. After completion of granule cell migration, AMOG becomes expressed by astrocytes in the internal granular layer, where it remains expressed in adulthood. That AMOG has a direct and selective role in adhesion is best indicated by its ability to bind to cells after purification: liposomes reconstituted with purified AMOG bind to cerebellar neurons and to pheochromocytoma PIC12 cells, but not to neurons of spinal cord or dorsal root ganglia (Antonicek and Schachner, 1988), whereas liposomes containing the myelin-associated glycoprotein (MAG) bind to spinal cord and dorsal root ganglion neurons but not to cerebellar neurons or PIC12 cells (Antonicek and Schachner, 1988; Poltorak et al., 1987; Sadoul et al., 1989). AMOG bears the carbohydrate epitope L3, which it shares with the adhesion molecules L1 and MAG (Kücherer et al., 1987). AMOG is an integral cell surface glycoprotein of 45,000–50,000 Mr, containing ~30% N-glycosidically linked carbohydrate (Antonicek et al., 1987). A 100,000-Mr component copurifies tenaciously with it, even in high salt or during FPLC in various detergents. Five tryptic peptides were derived from immunoaffinity-purified AMOG (including the 100K protein), purified by HPLC, and sequenced (Pagliusi et al., 1989). One of these was chosen for the design of an oligonucleotide probe, which was used to select a clone from a cDNA library from 8-d-old mouse brain mRNA. 54 bases of the resulting clone were sequenced; the sequence, which contained a stretch of nucleotides coding for the peptide, was not found in any previously identified gene. The β-galactosidase fusion protein reacted with antibodies raised against AMOG, and antibodies raised against the fusion protein reacted with AMOG itself, not the 100K associated protein. Northern blot analysis with the AMOG cDNA clone and ELISA analysis with monoclonal AMOG antibody revealed significant levels of AMOG and its mRNA in brain, but not in lung, liver, spleen, kidney, heart, or intest.
tine. All of these data were taken as evidence that the original clone was unique and specific for AMOG (Pagliusi et al., 1989).

In the present communication, the complete sequence of the AMOG cDNA has been obtained. Sequences coding for three of the original five tryptic peptides were found in the AMOG cDNA sequence, further confirming its identity. The striking outcome was that the AMOG sequence proved to be highly similar to the sequence of the subunit of the Na,K-ATPase, both in its length and the degree of nucleotide and amino acid similarity, and in the hydrophobicity profile and distribution of potential glycosylation sites.

Three different isozymes of the Na,K-ATPase subunit have been identified by genetic (Shull et al., 1986; Kent et al., 1987) and immunological evidence (Felsenfeld and Sweadner, 1988; Urayama et al., 1989; Shyjan and Levenson, 1989). The brain contains all three isofoms (Schneider et al., 1988; Urayama and Sweadner, 1988; Hsu and Guidotti, 1989), with complex and different cellular distributions (McGrail and Sweadner, 1989). Most remarkably, the two remaining unidentified AMOG-derived tryptic peptides proved to be found in the sequence of α2. Further evidence for the specific association of AMOG with the Na,K-ATPase is presented.

Materials and Methods

DNA Cloning and Sequence Analysis

Approximately 300,000 plaques of a lambda gt10 cDNA library established from 8- to 10-d-old mouse brain poly(A) + RNA (Moon et al., 1988) was screened with the previously described 32P-labeled partial AMOG clone 20k (Pagliusi et al., 1989). Plaque purification and cross-hybridization were carried out as described (Maniatis et al., 1982; Pagliusi et al., 1989). DNA sequence analysis was performed using the dideoxy chain termination procedure (Sanger et al., 1977). Deoxy- and dideoxy nucleotides for cDNA sequencing were from PL Biochemicals (Milwaukee, WI). Nucleic acid and protein sequence analysis were performed using PCgene (IntelliGenetics, Mountain View, CA), Microgenie (Beckman Instruments, Inc., Palo Alto, CA), and DNA, a program developed at the California Institute of Technology by A. L. Goldin (available from the National Institutes of Health-supported Molecular Biology Computer Research Resource of the Dana-Farber Cancer Institute, Harvard School of Public Health, Boston, MA).

Antibodies

Polyclonal AMOG antibodies were prepared in rabbits against mouse AMOG (including the 100K associated protein) which had been immunopurified as described by genetic (Antonicek et al., 1987). Monoclonal antibodies to AMOG and polyclonal antibodies to the neural cell adhesion molecule N-CAM, and the preparation of IgG fractions, have been described (Antonicek et al., 1987; Faisser and Sweadner, 1984). The antiseraum K3 was raised in rabbits against purified rat kidney Na,K-ATPase (Sweadner and Gilkeson, 1985). Monoclonal antibodies (AMOG-2F12 and AMOG-7C9) to the 100,000-M, associated protein in the AMOG antigen were prepared by immunization of rats with immunopurified AMOG from mouse brain. Fusion of splenocytes with mouse myeloma cells, screening of hybridoma supernatants, and cloning were performed as described previously (Poltorak et al., 1987). Monoclonal antibodies specific for the α1, α2, and α3 isofoms of the Na,K-ATPase (McKl, McB2, and Mcb3-X3, respectively) have been described (Felsenfeld and Sweadner, 1988; Urayama et al., 1989).

Western Blot Analysis

Purification of rat kidney Na,K-ATPase and rat brain Na,K-ATPase was by SDS extraction of contaminating proteins from membrane-bound enzyme (Sweadner and Gilkeson, 1985). Prestained relative molecular weight mark-
AMOG is inserted into the membrane with the carboxyl terminus facing the outside of the cell (Lipp and Dobberstein, 1986). Nine possible asparagine-linked glycosylation sites (Asn-X-Ser/Thr) were detected in the carboxyl-terminal portion of the protein (Fig. 1). One of these sites (NDS at amino acid position 118) was in one of the trypptic fragments sequenced by Pagliusi et al. (1989). The amino acid at the position of the asparagine in the peptide had not been determined unambiguously by protein sequencing, suggesting that this asparagine, in an otherwise correct peptide sequence, is most likely glycosylated in AMOG.

A striking structural feature of the deduced AMOG amino acid sequence is its 40% identity with the β polypeptide of the Na,K-ATPase from rat brain (Mercer et al., 1986; Young et al., 1987; Omori et al., 1988) (Fig. 2). Several gaps were permitted by hand for optimal alignment. Taking conservative amino acid exchanges into account, the similarity rose to ~56%. The similarity on the DNA level was ~50% as determined with Microgenie. Sequence alignment indicates that the amino acid similarity is fairly distributed along the entire sequence, with seldom more than four to five contiguous amino acids that are different. There are three places, however, where entire stretches of amino acid sequence appear to have been replaced with virtually unrelated sequences of different length (marked with brackets in Fig. 2). The structural similarity between AMOG and the Na,K-ATPase β subunit is further substantiated by the position of the seven cysteine domains in both proteins, with five of these residues being located in identical positions along the molecule and one being in a similar position (Fig. 2). Further comparisons of the two proteins at the level of secondary protein structure by hydropathy plots (Kyte and Doolittle, 1982) also revealed significant concordance (Fig. 3). The most hydrophobic peaks indicate the related positions of the putative transmembrane domains, suggesting that AMOG and the β polypeptide are inserted into the membrane in a similar way. It is noteworthy that the β polypeptide is also reported to lack a signal peptide sequence (Shull et al., 1986). Only one of the nine potential sites of N-linked glycosylation in AMOG was aligned with one of the three sites in the rat β subunit sequence.

Figure 1. Mouse brain AMOG cDNA sequence. Nucleotide and deduced amino acid sequence of the 1.1-kb clone. Nucleotides are numbered at the right. Amino acids are numbered starting with the amino-terminal methionine. The putative transmembrane region domain is indicated by the black bar and sequences of three tryptic peptides by double lines. Potential asparagine-linked glycosylation sites are labeled by asterisks.

The translated protein sequence consisted of 290 amino acids with a calculated relative molecular mass of 33,287 kD, which is in good agreement with the molecular mass (32,000 kD estimated by SDS gel electrophoresis) of AMOG synthesized in the presence of tunicamycin to block N-linked glycosylation (Antoneck et al., 1987). Sequences were found coding for three of the trypptic peptides isolated from purified AMOG (Pagliusi et al., 1989) (FVWN, FLEPYX-DSIQ, DEDAENLGH) (underlined by open bars in Fig. 1). The peptides were also preceded by lysines or arginines, except that FVWN was also preceded by a glutamate that had not been sequenced.

The initiation methionine was not followed by the typical signal peptide sequence expected for an integral membrane protein. A putative transmembrane domain was, however, found between residues 34 and 59 (Fig. 1), suggesting that

2. Because of a typographical error, the published sequence of this peptide had the last two amino acids inverted.

3. Proline is ~10% conserved in the rat/3 subunit sequence.

4. However, the asparagine in the peptide had not been determined.

5. In good agreement with the molecular mass (32,000 kD estimated by SDS gel electrophoresis) of AMOG synthesized in the presence of tunicamycin to block N-linked glycosylation (Antoneck et al., 1987).

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compared with purified mouse AMOG preparations includ-
ing the 100K associated protein (lanes 3, 4, and 5 in different
amounts). The blot in Fig. 4 A was stained with a rabbit an-
tiserum against rat kidney Na,K-ATPase, while the corre-
sponding blots in Fig. 4 B were stained with an antiserum
against the Na,K-ATPase/β subunits in Fig. 4 A, lanes 1 and 2 is thus a known
structural and functional association of AMOG
with the catalytic, α subunit of this enzyme was investigated.
The first piece of evidence came from the examination of the
sequences of the remaining two tryptic peptides reported in
Pagliusi et al. (1989), but not found in the sequence of the
AMOG gene (LDWDDR, YPGGWVE). Both proved to be
found in the sequence of the α2 subunit of the rat Na,K-
ATPase, preceded by arginines (Shull et al., 1986). In α1 and
α3, the corresponding sequences have some amino acid
replacements. This strongly suggests that the 100,000-Mr
protein that copurifies with AMOG is α2.

Further evidence came from the gel electrophoretic and
immunological comparison of purified AMOG and purified
Na,K-ATPases (Fig. 4). In Fig. 4, A and B, purified rat kid-
ney (lanes 1) and rat brain (lanes 2) Na,K-ATPases were
compared with purified mouse AMOG preparations including
the 100K associated protein (lanes 3, 4, and 5 in different
amounts). The blot in Fig. 4 A was stained with a rabbit an-
tiserum against rat kidney Na,K-ATPase, while the corre-
sponding blots in Fig. 4 B were stained with an antiserum
against mouse AMOG. (Monoclonal antibody 426 against
AMOG could not be used since it does not react with SDS-
denatured protein.) It can be seen first, that AMOG and its
associated protein and the subunits of brain Na,K-ATPase
were cross-reactive and of indistinguishable electrophoretic
mobilities. Kidney and brain Na,K-ATPases are known to
have β subunits with shared antigenic determinants but
different electrophoretic mobilities, ascribed at least in part
to differences in N-linked carbohydrate groups (Sweadner
and Gilkeson, 1985). The difference in mobility of the Na,K-
ATPase β subunits in Fig. 4 A, lanes 1 and 2 is thus a known
characteristic.
The reaction of the anti-Na,K-ATPase antisem with
AMOG and its associated protein in Fig. 4 A, lanes 3 and
4, was weak relative to the reaction of anti-AMOG antise-
rum with the same samples in Fig. 4 B, lanes 4 and 5. Com-
parable levels of staining of rat brain Na,K-ATPase and
mouse AMOG and its associated protein by anti-AMOG an-
tiserum were seen only when the concentration of the
AMOG sample was reduced to 0.5 μg/lane (Fig. 4 B, lane
3). This implies that rat brain Na,K-ATPase and mouse brain
AMOG and its associated protein have antigenic differences.
These may be due in part to species differences, but because
both antibodies reacted equally well with samples of crude
rat and mouse brain microsomes (data not shown), in all
likelihood the differences reflect the presence of mixtures of
different isoforms in different proportions. It is notable that
the anti-AMOG antisem reacted little if at all with the
purified rat kidney Na,K-ATPase, which is predominantly
α1 and β. AMOG mRNA is virtually undetectable in mouse
kidney (Pagliusi et al., 1989), and α2 mRNA has been de-
tected at only very low levels in rat kidney, if at all, in six
out of seven studies (reviewed in Sweadner, 1989).
The three isoforms for the Na,K-ATPase α subunit have
slightly different electrophoretic mobilities in SDS that can
be seen best in gels of 5% polyacrylamide; α1 migrates
fastest and α3 slowest (Schneider et al., 1988; Urayama et
al., 1989). This can be seen in Fig. 4 C, where the staining
of mouse AMOG-associated protein (lane 1), rat kidney
Na,K-ATPase (lane 2), and rat brainstem axolemma Na,K-
ATPase (lane 3) are compared with four different monoclo-
nal antibodies. Brainstem axolemma, a membrane prepara-
tion obtained from myelinated axons, has reduced levels of
α1 but contains both α2 and α3 (Hsu and Guidotti, 1989;
Urayama et al., 1989). A monoclonal antibody raised against
the AMOG-associated protein (Fig. 4 C) reacted with bands
in all three preparations, but the electrophoretic mobility of
the band in AMOG antigen was slightly lower than that in
kidney Na,K-ATPase, implying that different isoforms were
being detected. Protein mapping experiments have indicated
that this antibody reacts with all three isoforms of the Na,K-
ATPase (Sweadner, K. J., unpublished results). A monoclo-
nal antibody specific for α1 reacted with bands only in rat
kidney and axolemma Na,K-ATPases (Fig. 4 D); AMOG an-
tigen appeared to contain even less α1 than axolemma Na,K-
ATPase. A monoclonal antibody specific for α2 reacted with
bands only in AMOG antigen and axolemma Na,K-ATPase. A
monoclonal antibody specific for α3 but weakly cross-reactive with α1 stained a band
darkly in axolemma Na,K-ATPase, and stained lightly the α1 band
found in kidney Na,K-ATPase. It also stained lightly a band in AMOG antigen whose slower electrophoretic mobil-
ity suggests the presence of α3 rather than α1. All four of
these antibodies react with both mouse and rat antigens (Fel-
senfeld and Sweadner, 1988; Urayama et al., 1989; unpub-
lished observations). The implication is that the AMOG-
associated 100K protein is comprised predominantly of Na,K-
ATPase α2.

In view of the functional and molecular association be-
tween AMOG and the α subunit of Na,K-ATPase, it seemed
pertinent to investigate whether the two proteins are ex-
pressed in one and the same cell. Double immunofluores-
cence experiments on cultured astrocytes using polyclonal
AMOG antibodies and a monoclonal antibody directed
against the 100,000-Mr component of AMOG clearly showed
that both AMOG and the α subunit were coexpressed in a

**Figure 3.** Hydropathy plots of AMOG (top) and Na,K-ATPase β subunit (bottom). 15-residue averages have been plotted using the prediction algorithm of Kyte and Doolittle (Kyte and Doolittle, 1982). The putative transmembrane region is indicated by a black bar.
Figure 4. Immunological comparison of Na,K-ATPase and AMOG subunits. (A and B) Western blot analysis of antigen preparations of mouse AMOG (including associated 100K protein), and rat kidney and brain Na,K-ATPases on gels of 7.5% polyacrylamide. The antibodies used were: (A) polyclonal antibody to Na,K-ATPase from rat kidney (K3) at a dilution of 1:1,000; (B) polyclonal antibody to AMOG at a dilution of 1:400. The samples were: lane 1, rat kidney Na,K-ATPase (1 μg); lane 2, rat brain Na,K-ATPase (5.6 μg); lanes 3, 4, and 5, immunoaffinity-purified AMOG antigen in different amounts: (A) lane 3, 2 μg; lane 4, 10 μg; (B) lane 3, 0.5 μg; lane 4, 2 μg; lane 5, 10 μg. (C–F) Four identical blots of 5% polyacrylamide gels were prepared and stained with different monoclonal antibodies. In all blots, the samples were as follows: lane 1, majority, although not all, of these cells (Fig. 5). Similar results were obtained when McB2, a monoclonal antibody specific for the Na,K-ATPase α2 subunit, was used in conjunction with anti-AMOG antibodies (data not shown). The observation that the AMOG-7C9 antibody did not label all cells remains presently unexplained, but it could be due to different sensitivities of monoclonal α-antibody for the different isoforms, or to different sensitivities of the α isoforms to the fixation and permeabilization protocols.

Functional Link between Adhesion and Ion Transport

To investigate whether AMOG may be functionally linked to the α subunit of the Na,K-ATPase, the influence of AMOG antibodies on ion transport activity was measured on purified populations of cultured astrocytes, virtually all of which express glial fibrillar acidic protein (Antonicek et al., 1987) and most of which express AMOG (Fig. 5). Pump activity for K⁺ was determined by measuring uptake of 86Rb⁺ into cells as a function of time in the absence and presence of the monoclonal AMOG antibody 426, which reduces adhesion and which is known to react with the extracellular domain of the molecule (Fig. 6). In the presence of AMOG antibody the uptake of 86Rb⁺ was increased over control values obtained in parallel in the absence of antibody (set to 100%) (Fig. 6 A). The effect of AMOG antibody was specific since polyclonal N-CAM antibodies reacting with astrocytes did not show an effect at antibody concentrations known to interfere with various N-CAM-mediated functions in vitro (Fig. 6 A). The AMOG antibody-stimulated increase in 86Rb⁺ uptake was blocked by the cardiac glycoside ouabain, a specific inhibitor of Na,K-ATPase, at all time points (Fig. 6 A). At 5 min there was proportionally smaller effect of ouabain on basal 86Rb⁺ uptake. This may be due to a predominance of carrier- or channel-mediated fluxes at short times, or to the slow onset of inhibition by ouabain, a process that can take long times to approach equilibrium. The effect of ouabain was significant at 20 and 60 min and indicates that the effect of the AMOG antibody was mediated by the Na,K-ATPase.

Since we were aware that homology between an adhesion molecule and a subunit of an ion pump is unconventional, it seemed important to obtain evidence independent of the binding of AMOG-containing liposomes (Antonicek and Schachner, 1988) as to whether AMOG itself is the binding ligand in adhesion, or whether AMOG antibodies alter ion transport such that adhesion is indirectly affected in our assay system. Therefore, adhesion experiments were carried out at 4°C, a condition in which pump activities can no longer be detected, but in which recognition between adhesion molecules should still take place. At 4°C, only 50% of all probe cells adhered to the monolayer target cells, whereas at room temperature ~80% of all input cells attached to the AMOG antigen, 1 μg; lane 2, rat kidney microsomes, 5.6 μg; lane 3, rat brainstem axolemma, 5.9 μg. The blot labeled all α's was stained with a monoclonal antibody (AMOG-7C9) to the 100K component in the AMOG antigen preparation, at a dilution of 1:20. The blot labeled α1 was stained with monoclonal antibody to α1 (McK1) at a dilution of 1:20. The blot labeled α2 was stained with monoclonal antibody to α2 (McB2) at 1:20. The blot labeled α3 was stained with monoclonal antibody to α3 (McB-X3) at 1:10.
monolayer in 30 min. When neurons were allowed to adhere to astrocytes at 4°C in the presence of AMOG antibodies, an inhibition of adhesion of ~23% was seen (Table I) similar to the 26% inhibition observed previously at room temperature (Antonicek et al., 1987). At 4°C, inhibition of adhesion by Fab fragments of polyclonal N-CAM antibodies was only ~13% (Table I) as opposed to ~29% at room temperature (Keilhauer et al., 1985). Fab fragments of polyclonal antibodies to mouse liver membranes did not interfere with adhesion at any temperature (not shown; see also Antonicek et al., 1987).

Inhibition of the Na,K-ATPase with ouabain was also tested for an effect on adhesion. Cells were preincubated with the inhibitor for 15 min at 37°C to allow binding to the Na,K-ATPase. Ouabain did not interfere significantly with subsequent tests of adhesion either at 4°C or at room temperature (Table I). Furthermore, when adhesion tests were carried out at room temperature, ouabain did not influence the inhibition of adhesion by Fab fragments of monoclonal AMOG antibody 426 (Table I). These experiments further substantiate the notion that AMOG is an adhesion molecule in the strict sense of the operational definition, and further rule out the possibility that AMOG antibodies may have perturbed adhesion because of their influence on ion transport.

Discussion

AMOG Is Associated with the Na,K-ATPase

The original identification of AMOG as an adhesion mole-
cule used criteria that have been used for the identification of many characterized adhesion molecules to date: a specific antibody was found that blocked adhesion in in vitro assays. Inhibition in such assays is usually on the order of 30%, a value that reflects the complexity of the event and the presence of multiple adhesion components. The most compelling evidence that AMOG is an authentic adhesion molecule was that the purified protein, reconstituted into lipid vesicles, demonstrated selective adhesion to neurons in vitro (Antonicek and Schachner, 1988). Similar strict criteria have been applied only to N-CAM, L1, or MAG (Hoffman and Edelman, 1983; Sadoul et al., 1983; Poltorak et al., 1987; Kadmion et al., 1990; Sadoul et al., 1989), and many other candidate adhesion molecules have not yet been characterized to that extent. Our study has shown that the structure of AMOG differs from the Ca2+-independent adhesion molecules L1 (Moos et al., 1988), MAG (Arquint et al., 1987; Lai et al., 1987; Salzer et al., 1987), and N-CAM (Hemperly et al., 1986; Barthels et al., 1987), and the Ca2+-dependent adhesion molecules of the cadherin group (Takeichi, 1988). The protein sequence does not contain immunoglobulin-like domains, nor type III repeating unit fibronectin-like domains. It also bears no obvious similarity to a new family of endothelial leukocyte adhesion proteins containing lectin, EGF motif, and complement regulatory domains, and that is also not related to the immunoglobulin superfamily (reviewed in Stoolman, 1989). The only structural similarity, in fact, to many other known adhesion molecules, appears to be that the cDNA sequence predicts AMOG to be a membrane glycoprotein anchored by a transmembrane segment and a small cytoplasmic portion, with the majority of its mass exposed at the extracellular surface. It is unlikely that the carbohydrate attached to AMOG is more important than the protein in mediating adhesion, since the monoclonal AMOG antibody that blocks adhesion recognizes the unglycosylated apoprotein (Antonicek et al., 1987).

The striking observation of our study is that AMOG is structurally similar, but not identical to the β subunit of Na,K-ATPase, a glycoprotein of 45,000-50,000 M₉, whose function is not clearly known, but which appears necessary for the correct folding of the α subunit (Geering et al., 1989). Because the Na,K-ATPase β subunit is highly conserved among five different mammalian species (>85% identity at the protein level and three possible N-linked glycosylation sites) (Mercer et al., 1986; Shull et al., 1986; Kawakami et al., 1986; Brown et al., 1987; Ochinnikov et al., 1986), the β subunit and AMOG appear to be distinct, yet related proteins. This difference is substantiated by two observations: the genes for the β subunit and AMOG are localized on different mouse chromosomes (Kent et al., 1987; C.-L. Hsieh and U. Francke, personal communication), and the size and the expression pattern of the single mRNA coding for mouse AMOG (3 kb) (Pagliusi et al., 1989) clearly differs from the several mRNA sizes of the rat β subunit (1.25–2.55 kb) (Young et al., 1987).

Table 1. Adhesion of Neurons to Astrocytes in the Absence and Presence of AMOG Antibodies and Ouabain at 4°C and at Room Temperature

| Additive                          | Concentration | Percent inhibition of adhesion |
|----------------------------------|---------------|-------------------------------|
| AMOG antibodies, 4°C             | 0.5 mg/ml     | 23.5 (±7.6)                   |
| AMOG antibodies,room temperature | 1.0 mg/ml     | 26 (±1.8)                     |
| N-CAM antibodies, 4°C            | 0.5 mg/ml     | 13.2 (±6.1)                   |
| N-CAM antibodies, room temperature | 1.0 mg/ml     | 29 (±7)                       |
| Ouabain, 4°C                     | 1 mM          | 2.7 (±11.9)                   |
| Ouabain, room temperature        | 1 mM          | 2.4 (±8.7)                    |
| Ouabain plus AMOG antibodies, room temperature | 21.5 (±7.1) |

Antibodies or ouabain were allowed to bind to monolayer cultures of pure astrocytes before addition of single cell suspensions of pure small cerebellar neurons and incubation for 30 min at 4°C or room temperature. AMOG antibodies were added as Fab fragments of the monoclonal antibody. Values are means from three independent experiments carried out in triplicates or quadruplicates ± SD (in brackets).

* Taken from Antonicek et al. (1987).
† Taken from Keilbauer et al. (1985).
Martin-Vasallo et al. (1989) have independently cloned the same cDNA from human fetal liver and rat brain cDNA libraries using reduced stringency hybridization with a rat β subunit cDNA probe. Their objective was to find an alternative β subunit to explain the apparent absence of the conventional β subunit in certain tissues (reviewed in Pambrough, 1988; Sweadner, 1989; Shyjan and Levenson, 1989). The only evidence they had that the cDNA (termed β2) was in any way associated with the Na,K-ATPase, however, was its sequence homology. The sequence of the rat β2 cDNA differs from the mouse AMOG cDNA at only nine amino acids, consistent with species differences. In agreement with the results of Pagliusi et al. (1989), Martin-Vasallo et al. (1989) observed that the brain has the highest concentration of the mRNA. Martin-Vasallo et al. (1989), however, observed low levels of β2 mRNA hybridization in kidney, lung, and heart, where Pagliusi et al. (1989) did not detect it. Recent experiments show that this discrepancy is due to different exposure times: although the difference in relative abundance is very great, with long exposure times low levels of AMOG probe hybridization can be detected in the other tissues (Gloor, S., unpublished observations). Martin-Vasallo et al. (1989) also observed the same clear difference in the sizes of the β and β2 mRNAs.

The evidence presented in this paper indicates that AMOG (β2) is indeed tightly associated with the Na,K-ATPase, since it was seen to copurify with the α2 subunit both by immunoaffinity chromatography in detergent, and by Na,K-ATPase purification by extraction of membranes with SDS (Fig. 4). Immunoaffinity-purified AMOG has been observed to contain a variable amount of the 100K associated protein (Antonicek et al., 1987; Antonicek and Schachner 1988; and ongoing experiments). The antibody used for the affinity column is directed against AMOG, and the 100K protein is not always recovered in stoichiometric amounts. This is as might be expected for two hydrophobic proteins that are held together only by noncovalent bonds, isolated in detergent. Similar observations were made by Fambrough and Banyei (1983), whose monoclonal antibody (Ab 24) was specific for a β subunit of the Na,K-ATPase. Their work is particularly relevant in that their antibody recognized the Na,K-ATPase of muscle cells but failed to recognize the Na,K-ATPase of fibroblasts, despite an equivalent number of ouabain binding sites. This was among the earliest suggestions that there may be more than one type of β subunit.

It is not yet known whether AMOG (β2) always associates only with α2 (and α3), or whether it associates with α1 in other tissues or at other developmental ages. The fact that Martin-Vasallo et al. (1989) obtained the corresponding cDNA from a human liver library suggests that it might, since only α1 has yet been detected in liver. AMOG expression has already been demonstrated to be developmentally regulated (Antonicek et al., 1987). The three Na,K-ATPase α subunit isoforms are also developmentally regulated (Orlowski and Lingrel, 1988; Urayama et al., 1989), but their distributions have not yet been studied in sufficient cellular detail to permit comparison.

While evidence is presented here that AMOG (β2) is associated with the Na,K-ATPase, no claim is made that it is equivalent to and interchangeable with the conventional β subunit. While it is logical to suppose that AMOG (β2) replaces β in association with the α subunit, this has not yet been actually demonstrated. It has also not yet been directly demonstrated that the α2/AMOG (β2) complex is enzymatically active. Considerably more biochemical investigation of the structure and function of the complex will be required.

Theoretical Implications of the Observations
Our observations on the association of an adhesion molecule with an ion pump have interesting implications. Either AMOG has two quite different roles to play at the cell surface, or there is a hitherto unknown linkage between cell recognition and the regulation of the ionic milieu. Adhesion is a process in which recognition (initial binding) is followed by other intracellular events relevant to morphogenesis. Some interactions result in adhesion; others are antiadhesive. Binding to the hypothetical AMOG receptor may directly modulate Na,K-ATPase activity, or the interaction of AMOG with its receptor may influence the enzyme through indirect means, such as an effect on its turnover. The activities of both Na+ and K+ are known to determine the membrane potential of cells, the size of the extracellular space, and cell volume (Sykova et al., 1983; Lux et al., 1986). The manner by which AMOG might modulate these features could be by either stimulating or reducing ATPase activity. It is also tempting to speculate that AMOG-mediated morphogenetic events, such as granule cell neuron migration along Bergmann glial cells in the developing cerebellar cortex (Antonicek et al., 1987; Rakic, 1971) may result from the dynamic interplay between adhesion and changes in intracellular ionic balance. A strong expression of AMOG in the internal granular layer of the adult cerebellar cortex after cessation of granule cell migration (Antonicek et al., 1987) may indicate that cell contacts between neurons and glia remain instrumental in regulating the ionic symbiosis between neurons and glia in the adult. Furthermore, it is interesting to speculate that determination of whether cell surface recognition molecules are adhesive or antiadhesive may result from their influence on different transducing systems in the form of ion pumps, channels, or carriers. The fact that a monoclonal AMOG antibody stimulates the pump activity of the Na,K-ATPase can be interpreted in several ways. It unambiguously indicates that the antibody is capable of interacting with active Na,K-ATPase, but whether this interaction mimics a biologically relevant phenomenon is not yet clear. A possibility is that the AMOG antibody mimics the postulated neuronal AMOG receptor and that the AMOG–receptor complex directly stimulates pump activity. Another possibility is that the AMOG antibody interferes with the interaction of AMOG with the α polypeptide, permitting the β subunit of the Na,K-ATPase to interact more freely with the α subunit, resulting in overall stimulation of pump activity. The stimulation could, however, result from an increase in insertion or decrease in recycling of the pump from the cell surface. It remains to be seen whether AMOG affects the functional interactions between the α and β subunits of the Na,K-ATPase.

It should be stressed that the role of the glycoprotein subunit of the Na,K-ATPase has never been explicitly defined. The structure of the β subunit is thought to be entirely different from that of the α subunit: β appears to be a type 1 membrane protein (anchored by a single transmembrane segment, typical of many recognition molecules), while the
α subunit is predicted to be a type 2 membrane protein (containing at least seven transmembrane folds, typical of other ion pumps and channels). Other members of the aspartyl-phosphate ATPase gene family do not appear to have a comparable β subunit. Emerging evidence suggests that the β subunit is necessary for the targeting of the α subunit to the plasma membrane (Takeyasu et al., 1988). Perhaps in this context it is not unreasonable to suppose that it may be enlisted to perform two functions. It also remains to be seen whether the suspected heterogeneity in the structure of β subunits may be due to AMOG-like molecules, contributing to the functional diversification of the Na,K-ATPase.

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References

Antonicek, H., and M. Schachner. 1988. The adhesion molecule on glia (AMOG) incorporated into lipid vesicles binds to subpopulations of neurons. J. Neurosci. 8: 2961–2966.

Antonicek, H., E. Persohn, and M. Schachner. 1987. Biochemical and functional characterization of a novel neuron-glial adhesion molecule that is involved in neuronal migration. J. Cell Biol. 104:1587–1595.

Arquint, M., J. Roder, L. S. Chia, J. Down, D. Wilkinson, H. Bayley, P. Braun, and R. Dunn. 1987. Molecular cloning and primary structure of myelin-associated glycoprotein. Proc. Natl. Acad. Sci. USA. 84:600–604.

Barthels, D. M., J. Santoni, W. Wille, C. Ruppert, J. C. Chais, M. R. Hirsch, J. C. Fontemilla-Camps, and C. Goridis. 1987. Isolation and nucleotide sequence of mouse NCAM cDNA that codes for a M, 79,000 polypeptide without a membrane-spanning region. EMBO (Eur. Mol. Biol. Organ.) J. 6:907–914.

Blake, M. S., K. H. Johnston, G. J. Russell-Jones, and E. C. Gotschlich. 1984. A rapid, sensitive method for detection of alkaline-phosphatase-conjugated anti-antibody of Western blots. Anal. Biochem. 136:175–179.

Bradl, M., M. Moos, R. Dunn, and K. J. Sweadner. 1988. A rapid and sensitive method for the quantitation of micromolar quantities of protein using the principle of protein-dye binding. Anal. Biochem. 72:248–254.

Brown, T. A., B. Horowitz, R. P. Miller, A. A. McDonough, and R. A. Farley. 1987. Molecular cloning and sequence analysis of the (Na+,K+) ATPase beta subunit from dog kidney. Biochem. Biophys. Acta. 912:244–253.

Faissner, A., J. Kruse, C. Goridis, E. Bock, and M. Schachner. 1984. The neuromuscular junction: structural and functional characterization of a novel neuron-glia adhesion molecule that is involved in neuronal migration. J. Cell Biol. 104:1587–1595.

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Schnitzer, J., and M. Schachner. 1981. Characterization of isolated mouse cerebellar cell populations in vitro. J. Neuroimmunol. 1:429-456.
Shull, G. E., J. Greeb, and J. B. Lingrel. 1986a. Molecular cloning of three distinct forms of the Na⁺, K⁺-ATPase α-subunit from rat brain. Biochemistry. 25:8125-8132.
Shull, G. E., L. K. Lane, and J. B. Lingrel. 1986b. Amino-acid sequence of the β-subunit of the (Na⁺ + K⁺)ATPase deduced from a cDNA. Nature (Lond.). 321:429-431.
Shyjan, A. W., and R. Levenson. 1989. Antiserum specific for the alpha 1, alpha 2, alpha 3, and beta subunits of the Na,K-ATPase: differential expression of the alpha and beta subunits in rat tissue membranes. Biochemistry. 28:4531-4535.
Stoolman, L. M. 1989. Adhesion molecules controlling lymphocyte migration. Cell. 56:907-910.
Sweadner, K. J. 1989. Isozymes of the Na,K-ATPase. Biochim. Biophys. Acta. 988:185-220.
Sweadner, K. J., and R. C. Gilkeson. 1985. Two isozymes of the Na,K-ATPase have distinct antigenic determinants. J. Biol. Chem. 260:9016-9022.
Sykova, E. 1983. Extracellular K⁺ accumulation in the central nervous system.

Progr. Biophys. Mol. Biol. 41:135-189.
Takeichi, M. 1988. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. Development. 102:639-655.
Takeyama, K., M. M. Tamkun, K. J. Renaud, and D. M. Fambrough. 1988. Ouabain-sensitive Na,K-ATPase activity expressed in mouse L cells by transfection with DNA encoding and alpha-subunit of an avian sodium pump. J. Biol. Chem. 263:4347-4354.
Trotter, J., D. Bitter-Suermann, and M. Schachner. 1989. Differentiation-regulated loss of the polysialylated embryonic form and expression of the different polypeptides of the neural cell adhesion molecule (N-CAM) by oligodendrocytes and myelin. J. Neurosci. Res. 22:369-383.
Urayama, O., and K. J. Sweadner. 1988. Ouabain sensitivity of the alpha 3 isozyme of rat Na,K-ATPase. Biochem. Biophys. Res. Commun. 156:796-800.
Urayama, O., H. Shutt, and K. J. Sweadner. 1989. Identification of three isozyme proteins of the catalytic subunit of the Na,K-ATPase in rat brain. J. Biol. Chem. 264:8271-8280.
Young, R. M., G. E. Shull, and J. B. Lingrel. 1987. Multiple mRNAs from rat kidney and brain encode a single Na⁺,K⁺-ATPase β subunit protein. J. Biol. Chem. 262:4905-4910.