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Specific Antibodies Against G_o Isoforms Reveal the Early Expression of the $G_{o_{2\alpha}}$ Subunit and Appearance of $G_{o_{1\alpha}}$ During Neuronal Differentiation

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SUMMARY

We have previously identified two isoforms of $G_{o\alpha}$ in membranes of N1E-115 neuroblastoma cells, using an antibody raised against the purified $G_{o\alpha}$ subunit; one isoform of the $G_{o\alpha}$ subunit (pI 5.80) is present in undifferentiated cells, whereas a more acidic isoform (pI 5.55) appears during differentiation [*J. Neurochem.* **54**:1310–1320 (1990)]. Recently, the $G_{o\alpha}$ gene has been shown to encode, by alternative splicing, two polypeptides, $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$, which differ only in their carboxyl-terminal part. To determine unambiguously whether the two $G_{o\alpha}$ subunits detected in neuroblastoma cells were actually the products of different mRNAs, rabbit polyclonal antibodies were generated against synthetic peptides (amino acids 291–302) of both sequences. Specificity of the two affinity-purified antipeptide antibodies was assessed on Western blots by comparing their immunoreactivities with those of other G_α antibodies. On a blotted mixture of purified brain guanine nucleotide-binding proteins, the anti-α_{o_1} and anti-α_{o_2} peptide antibodies only recognized the 39-kDa $G_{o\alpha}$ subunit. Furthermore, the immunological recognition of brain membranes from 15-day-old mouse fetuses by antipeptide antibodies could be specifically blocked by addition of the corresponding antigen. When membrane proteins from differentiated neuroblastoma cells and mouse fetus brain were blotted after two-dimensional gel electrophoresis, the anti-α_{o_1} and anti-α_{o_2} peptide antibodies labeled a 39-kDa subunit focused at a pI value of 5.55 or 5.80, respectively. Study of the ontogenesis of both $G_{o\alpha}$ subunits revealed the predominance of $G_{o_{2\alpha}}$ in the frontal cortex at day 15 of gestation. Thereafter, there was a progressive decline of the $G_{o_{2\alpha}}$ polypeptide to a very low level, concomitant with an increase in the $G_{o_{1\alpha}}$ protein, which plateaued about 15 days after birth to a level 8 times higher than at gestational day 15. Similarly, on neuroblastoma cells, the $G_{o_{2\alpha}}$ subunit was almost exclusively present in undifferentiated cells, and differentiation induced the appearance of the $G_{o_{1\alpha}}$ subunit, with a reduction in the amount of $G_{o_{2\alpha}}$ polypeptide. Thus, the evolution of the two $G_{o\alpha}$ subunits during cell differentiation, unambiguously identified with specific antibodies, suggests that neuronal differentiation is responsible for the on/off switch of the expression of the $G_{o\alpha}$ isoforms and indicates that $G_{o_{1\alpha}}$, rather than $G_{o_{2\alpha}}$, is involved in neurotransmission.

G proteins are a family of regulatory proteins linking cell surface receptors with ion channels or intracellular enzymes (for reviews, see Refs. 1 and 2). Each of these transducing G proteins is a heterotrimer composed of α, β, and γ subunits, with a GTP binding site and GTPase activity located on the α subunit. It is believed that selectivity of the transmembrane signaling function mainly resides in differences that exist between individual α subunits, although a certain degree of genetic diversity has recently been shown for the β and γ subunits (3, 4). Multiplicity of α subunits was first identified among G proteins sensitive to ADP-ribosylation, which, according to subtype, can be catalyzed either by cholera toxin (four forms of $G_{s\alpha}$), pertussis toxin (four forms of $G_{i\alpha}$ and $G_{o\alpha}$), or both toxins (two forms of transducins). The family of pertussis toxin-sensitive G proteins is composed of three different $G_{i\alpha}$ polypeptides (named G_{i_1}, G_{i_2}, and G_{i_3}), plus another $G_{o\alpha}$ subunit that is present mainly in the central nervous system (5–7). Due to its abundance in brain, large quantities of G_o protein have been purified, which has enabled full biochemical characterization of this α subunit as well as many reconstitution experiments with receptors. However, during the course of purification, heterogeneity of the $G_{o\alpha}$ subunit was observed (8, 9). Goldsmith *et al.* (8) were thus able to purify substantial amounts of another form of G_o (referred to as G_o*), whereas Inanobe *et al.* (10), using a quaternary amine ion exchange column, identified as many as four different entities recognized by antibodies against a specific $G_{o\alpha}$ peptide.

Thus, it was unclear whether these multiple isoforms of $G_{o\alpha}$ were products of differential splicing of nuclear RNA tran-

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; PVDF, polyvinylidene difluoride; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectrofocusing.

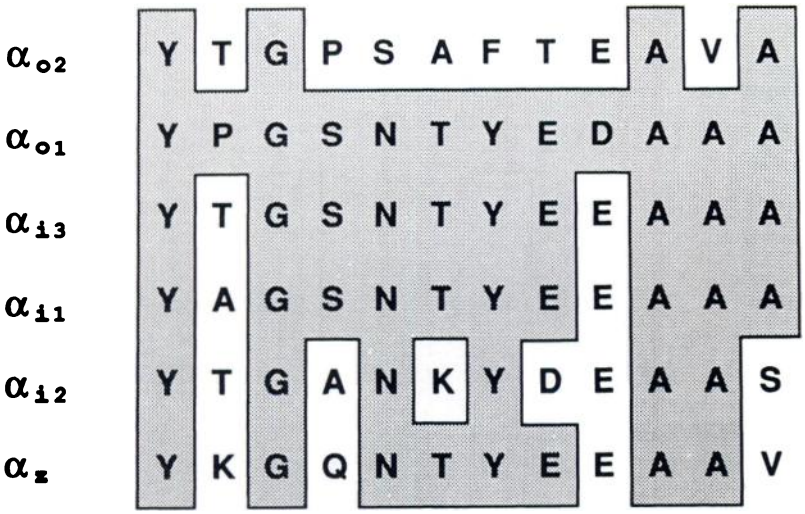

Fig. 1. Comparison of the sequences of amino acids 291–302 deduced from α_{o_1} and α_{o_2} cDNAs with the corresponding domain of other G proteins. Amino acid residues are designated by standard one-letter symbols. Amino acid residues that, in each column, are identical to that of α_{o_1} are included in the *shaded box*. Sequences presented are those deduced from rat cDNAs, except for α_{o_2}, which was cloned from only mouse and hamster cDNAs. The corresponding α_s or α_q predicted sequences, with less than five amino acids identical to either α_{o_1} or α_{o_2} sequences, are not shown.

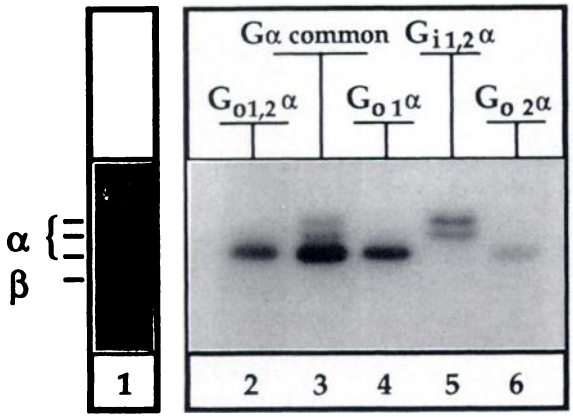

Fig. 2. Determination of the specificity of the $G_{o_1\alpha}$ and $G_{o_2\alpha}$ antibodies using purified G proteins. A mixture of G_o, G_{i_1}, and G_{i_2} purified from bovine brain was treated with NEM as previously described (19). Mixtures of purified G proteins were separated by electrophoresis over a 30-cm-long gel and were stained with Coomassie blue either directly (*lane 1*) (6 μg) or after electrotransfer onto a PDVF sheet (*lanes 2–6*) (2 μg each). Strips, corresponding to individual tracks, were then cut from the PDVF sheet and immunostained, as indicated in Materials and Methods, by incubation with the anti-$G_{i_{1,2}\alpha}$ serum at a 1/200 dilution (*lane 2*) or with the affinity-purified antipeptide antibodies anti-α common (*lane 3*), anti-$G_{o_1\alpha}$ (*lane 4*), anti-$G_{i_{1,2}\alpha}$ (*lane 5*), and anti-$G_{o_2\alpha}$ (*lane 6*), at concentrations ranging from 8 to 20 μg/ml. The immune complexes were visualized using iodinated Protein A and autoradiography. The figure shows the relevant portion of the autoradiogram of strips (*lanes 2–6*) that were exposed for 3 days without an intensifying screen.

scribed from a single gene or whether they represented different posttranslationally modified isoforms of the same polypeptide. The reality of multiple $G_{o\alpha}$ isoforms was suggested by the fact that cell differentiation could specifically induce the expression of one $G_{o\alpha}$ isoform. Thus, blots from a two-dimensional electrophoresis gel of proteins from undifferentiated neuroblastoma cells exhibited a single $G_{o\alpha}$ immunoreactive polypeptide, with a pI value close to 5.80 (11). We have shown that differentiation produced by dimethyl sulfoxide induced another $G_{o\alpha}$ isoform, with a pI value of about 5.55. Similar results were later obtained by Mullaney and Milligan (12), using neuroblastoma × glioma hybrid NG 108-15 cells. Moreover, these authors indicated that, besides these two $G_{o\alpha}$ isoforms, there was another G_o form, which might correspond to the $G_{o\alpha}$* previously purified from bovine brain by Goldsmith *et al.* (8).

The existence of at least three individual species of $G_{o\alpha}$ mRNA transcripts (13) that can be identified on Northern blots using $G_{o\alpha}$-specific probes also strengthened the evidence for possible diversity among amino acid sequences of the various $G_{o\alpha}$ isoforms. In agreement with this observation, a $G_{o\alpha}$ mRNA variant, produced by an alternative splicing from the same $G_{o\alpha}$ gene, has been demonstrated recently (14, 15). This mRNA encodes another $G_{o\alpha}$ subunit of the same length but with differences in the carboxyl-terminal part. Thus, there are now two forms of the $G_{o\alpha}$ subunit; the first to be identified was named $G_{o_1\alpha}$ (14) or $G_{o_{A\alpha}}$ (15), whereas the more recently identified form was called $G_{o_2\alpha}$ (14) or G_{o_B} (15), depending on the authors. Examination of the peptide sequences previously used as antigens to generate specific $G_{o\alpha}$ antibodies (16, 17), i.e., from the amino terminus or the very end of the carboxyl terminus, indicates that these antibodies could not discriminate between the $G_{o_1\alpha}$ and $G_{o_2\alpha}$ sequences.

The purpose of this study was to assess clearly to which sequence (deduced from the two α_o cDNAs) either $G_{o\alpha}$ isoform corresponds. We report here the characterization of specific antibodies towards peptides specific to the $G_{o_1\alpha}$ and $G_{o_2\alpha}$ sequences. In neuroblastoma cells, we were able to demonstrate that the early expressed isoform of $G_{o\alpha}$ (pI 5.8) is recognized by the antibodies raised against the α_{o_2} peptide, whereas the isoform at pI 5.5, appearing after differentiation, displays immunoreactivity towards anti-α_{o_1} peptide antibodies. We also show that the same two $G_{o\alpha}$ isoforms occur in mouse brain development. Thus, $G_{o_2\alpha}$ is present in fetuses at day 15 (G_{15}) and progressively declines thereafter. Reciprocally, the $G_{o_1\alpha}$ isoform, which is undetectable or at low levels at G_{15}, gradually increases, to reach its maximal level about 15 days after birth.

Materials and Methods

Preparation of affinity-purified antibodies. Two dodecapeptides, taken from the same region of $G_{o_1\alpha}$ and $G_{o_2\alpha}$ sequences, were synthesized using benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate and butyloxycarbonyl-protected amino acids, on a 4-methylbenzydrylamine resin (Nova-Biochem, Switzerland), which was first coupled with a cysteine residue. After hydrofluoric acid treatment, the resulting peptides, YPGSNTYEDAAA(C) and YTGPSAFTEAVA(C), were coupled to porcine thyroglobulin. Linking was performed either through the amino-terminal tyrosine, with glutaraldehyde (8), or with the sulfhydryl groups of the carboxyl-terminal

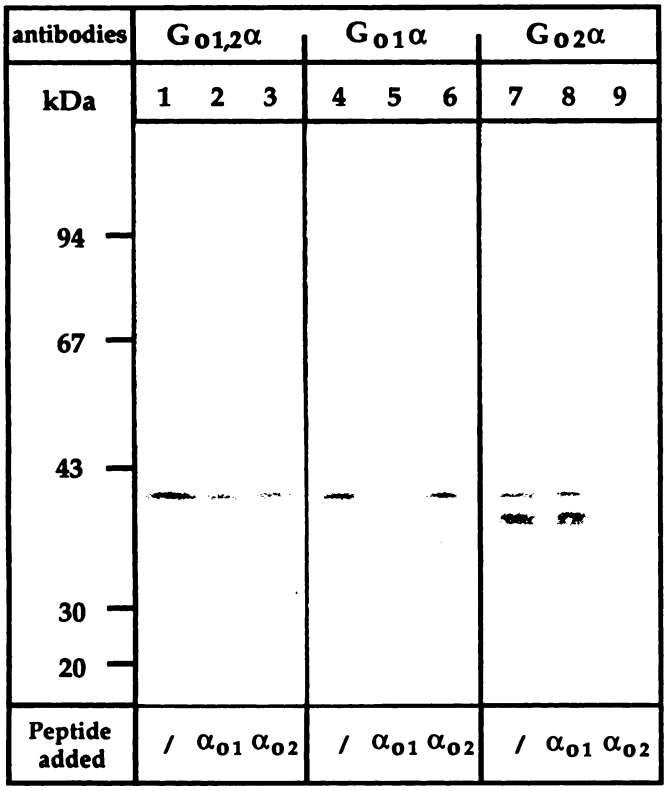

Fig. 3. Effect of the specific α_{o_1} and α_{o_2} peptides on the immunoreactivities of the different $G_{o\alpha}$ antibodies towards blotted mouse fetus brain proteins. Membranes from brain of 15-day-old mouse fetuses were electrophoresed, blotted onto PVDF sheets, and stained with Coomassie blue, as described in Materials and Methods. Strips that corresponded to the migration in the whole separating gel of the proteins loaded in each well were cut. Quenched strips were then incubated either with anti $G_{o_{1,2}\alpha}$ serum (*lanes 1–3*) at a 1/200 dilution or with 8–13 μg/ml specific $G_{o_1\alpha}$ or $G_{o_2\alpha}$ affinity-purified antibodies (*lanes 4–6* and *7–9*, respectively). The various antibody solutions had been previously preincubated for 1 hr at room temperature in the absence of added peptide (*lanes 1*, *3*, and *7*) or in the presence of 1 μg/ml α_{o_1} or α_{o_2} peptides (*lanes 2*, *4*, and *8* and *lanes 3*, *6*, and *9*, respectively). The figure shows the autoradiogram of strips after staining with ^{125}I-Protein A and 24-hr exposure without an intensifying screen.

cysteine, using the heterofunctional reagent succinylmaleimidocyclohexyl (Pierce, Netherlands), according to the manufacturer's recommendations. Four antigens were obtained, and each was injected into one hybrid from New Zealand white rabbits (HY 278; Elevage Scientific des Dombes, France), as previously described (18). Preliminary immunoblotting experiments indicated that the recognition ability of the anti-$G_{o_1\alpha}$ serum was independent of the method of coupling, whereas the best anti-$G_{o_2\alpha}$ antibodies were obtained with the α_{o_2} peptide linked through the carboxyl-terminal cysteine. Thus, the antibodies used in this study were those obtained by immunization with the α_{o_1} peptide linked to the carrier by the amino-terminal part and with the α_{o_2} peptide coupled by the cysteine residue. Antibodies were affinity purified by a two-step procedure. First, the ammonium sulfate-precipitated fraction from immune serum was recycled overnight through an Affi-gel 10 (Bio-Rad) column coupled with the other peptide, i.e., α_{o_2} peptide for the anti-$G_{o_1\alpha}$ antibodies and vice versa. Second, the antibodies present in this flow-through were retained on an Affi-gel 10 column coupled to the peptide used as an antigen. After acidic elution, neutralized affinity-purified antibodies were dialyzed against phosphate-buffered saline and then against a glycerol/phosphate-buffered saline (50:50) solution before storage at −25°. For comparison, three other antibodies were used, (a) those directed against GAGESGKSTIVK, a sequence common to all toxin-sensitive G proteins, (b) those directed against KNNLKDCGLF, the identical carboxyl-terminal part of the $G_{i_1\alpha}$ and $G_{i_2\alpha}$ subunits, and (c) those raised against the purified calf brain $G_{o\alpha}$ subunit, which recognized various isoforms of this protein and were termed throughout this study anti-$G_{o_{1,2}\alpha}$ antibodies (18).

Isolation of purified G proteins and particulate fraction. A mixture of G proteins composed of G_{i_1}, G_{i_2}, and G_o was purified from bovine brain as described previously (18). Particulate fractions were prepared from fetuses and the newborn of Swiss albino mice litters. The whole brain or dissected areas (cerebella, striata, colliculi, and frontal cortex) were homogenized with 15 strokes in a Potter-Elvehjem homogenizer, in buffer containing 50 mM Tris·HCl, pH 7.5, 3 mM EDTA, 5 μg/ml soybean trypsin inhibitor, and 0.1 mM freshly prepared phenylmethylsulfonyl fluoride. Particulate and soluble fractions were separated by centrifugation for 15 min at 40,000 × *g* in a Beckman JA 20 rotor. Particulate pellets were suspended in the same buffer (2–5 mg of protein/ml) and stored at −80° before use. Neuroblastoma cells were grown in OptiMEM medium (GIBCO) with 10% fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin, at 37°, in a humidified atmosphere with 10% CO_2. When necessary, cells were induced by 1.5% dimethyl sulfoxide to differentiate for various times in the presence of 0.5% fetal calf serum, as described previously (11). Cells were gently washed three times at room temperature with 0.15 M NaCl, scraped off with a rubber spatula into 0.15 M NaCl (10 ml/dish), and centrifuged for 10 min at 300 × *g*. Cell pellets were homogenized in 50 mM Tris·HCl, pH 7.5, 3 mM EDTA, 5 μg/ml soybean trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride, and then centrifuged for 15 min at 40,000 × *g*. Each homogenate was resuspended in the same buffer, at a final concentration of about 1–2 mg/ml of protein, and stored at −80° until use.

Immunoblotting of one-dimensional gel electrophoresis. Before one-dimensional electrophoresis, the samples were subjected to NEM treatment as already described (19). Briefly, 20 μl (27 μg) of the G protein mixture or 250 μl (1.2 mg) of mouse fetal brain membranes were heated for 5 min at 90° with 20 μl and 50 μl, respectively, of a solution composed of 20% SDS, 50 mM dithiothreitol, and 62 mM Tris·HCl. After cooling, each sample received an equivalent volume (20 or 50 μl) of 125 mM NEM, followed 15 min later by 60 or 300 μl, respectively, of Laemmli sample buffer. After boiling, about 2 μg (10 μl) of the G protein mixture or 37 μg (20 μl) of fetal brain membranes were loaded into several wells for separation by SDS-PAGE on 10% polyacrylamide/0.13% bisacrylamide gels. Gels were then electrotransferred onto a PDVF sheet (Immobilon, Millipore) and stained with Coomassie blue. Strips corresponding to each track were cut and destained. After quenching and washing, strips were incubated at 25° for 2 hr or at 4° overnight with the different antibodies. When indicated (see figure legends), the antibody solutions were preincubated for 1 hr at 30° in the presence or absence of 1 μg/ml α_{o_1} or α_{o_2} peptides. Antibody dilutions were 1/200 for the anti-$G_{o_{1,2}\alpha}$ serum, and the concentration of the affinity-purified antipeptide antibodies ranged from 8 to 20 μg/ml, as indicated in the figures. Labeling of the immune complexes was performed by a 1-hr incubation at 30° with ^{125}I-Protein A (2–3 × 10^5 cpm/ml) (New England Nuclear), followed by autoradiography without an intensifying screen.

Two-dimensional electrophoresis and immunoblotting. Membrane proteins (50 μg) were subjected to two-dimensional analysis as previously described (11). Proteins that had been separated on SDS-polyacrylamide gels were transferred onto nitrocellular sheets and immunolabeled using the dilutions and Protein A as described above. For blots with neuroblastoma particulate fractions, immune complexes were revealed with a 1/250 dilution of antiserum coupled to alkaline phosphatase (Miles ICN), according to the method of Ey and Ashman (20).

During differentiation of neuroblastoma cells and development of brain areas, samples were analyzed after electrotransfer of SDS-PAGE gels onto nitrocellulose sheets. At each time, the $G_{o\alpha}$ levels (sum of the two $G_{o\alpha}$ proteins) were determined using SDS-PAGE after incubation with anti-$G_{o_{1,2}\alpha}$ antiserum and ^{125}I-Protein A, by excision of labeled

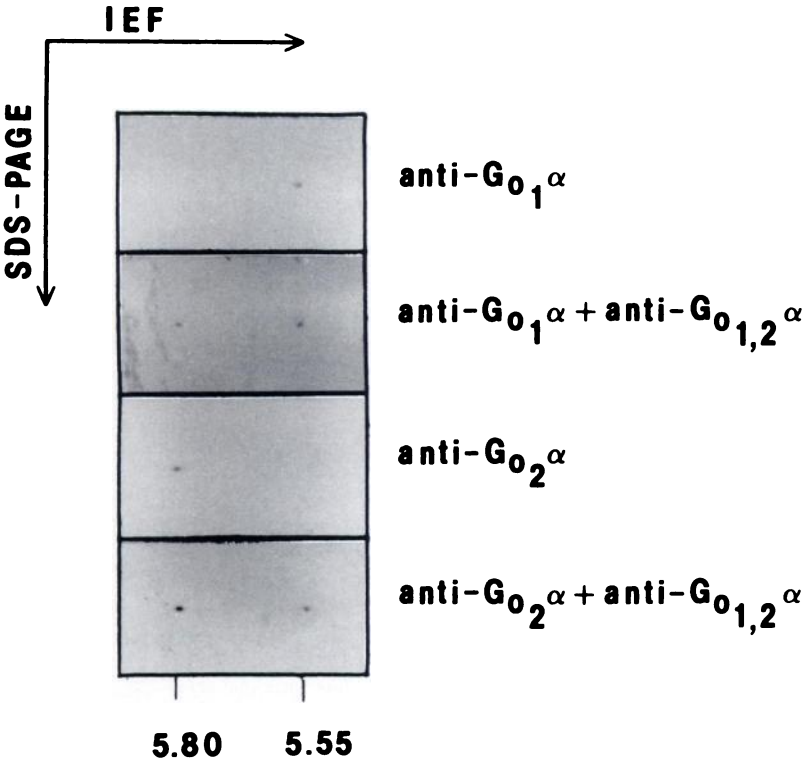

Fig. 4. Identification of G_{o_1} and $G_{o_{2\alpha}}$ in differentiated N1E-115 neuroblastoma cells. Particulate fractions (50 μg of protein) from 72-hr differentiated neuroblastoma cells were subjected to two-dimensional gel analysis and immunoblotting with affinity-purified anti-$G_{o_{1\alpha}}$ antibody (8 μg/ml), anti-$G_{o_{2\alpha}}$ antibody (13 μg/ml), or crude antiserum raised against purified bovine brain $G_{o\alpha}$ (anti-$G_{o_{1,2\alpha}}$, 1/200 dilution), followed by anti-IgG antibody coupled to alkaline phosphatase, as described in Materials and Methods. Only the relevant portions of blots are shown. When two different antibodies were used, treatment of blots was successive; first they were treated with specific antipeptide antibody and the immunostaining was developed and second, after staining, blots were processed with anti-$G_{o_{1,2\alpha}}$ antiserum. Only two isoforms of $G_{o\alpha}$ were detected, which focused at pH 5.80 and 5.55 after this double processing.

bands and measurement of radioactivity in a scintillation counter. The same samples were analyzed by two-dimensional electrophoresis, and the two $G_{o\alpha}$ proteins were detected using anti-$G_{o_{1,2\alpha}}$ antiserum followed by ^{125}I-Protein A. The ratio between the two $G_{o\alpha}$ proteins was determined by excising the spots from the blot and measuring the ^{125}I radioactivity and/or by quantitating the intensity of the spots from the autoradiogram using a Joyce-Loeb laser densitometer. The two methods gave similar results. To determine the $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$ levels, this two-step procedure was used instead of direct scanning of the intensity of spots after two-dimensional analysis, because in some samples 10–20% of the material did not enter the gel after electrofocusing in the first dimension. Thus, we assume that this material remaining at the top of the isofocusing tube did not significantly alter the ratio between the two $G_{o\alpha}$ isoforms.

Results

Delineation of the specificity of the $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$ antibodies. Polyclonal rabbit antibodies were generated from two synthetic peptides (α_{o_1} and α_{o_2} peptides) that correspond to the domains of the $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$ polypeptides that bear least similarity, i.e., amino acids 291–302 (Fig. 1). There is no homology between the α_{o_1} or α_{o_2} peptides and the α_s or α_q subunits (21), but comparison of the α_{o_2} peptide with the corresponding sequences of $G_{o_{1\alpha}}$ or other α subunits reveals positional identity in four or six amino acids of the 12 present in the chosen sequence. Furthermore, homology between the α_{o_1} peptide and the corresponding sequence of α_z and $\alpha_{i_1}/\alpha_{i_3}$ subunits is important, because 8–10 of the 12 amino acids are identically positioned (Fig. 1).

Because of this high degree of homology, the immunological specificity of both anti-α_{o_1} and anti-α_{o_2} peptide antibodies was first assessed using a mixture of purified bovine G proteins that were separated by resolving electrophoresis before electroblotting (Fig. 2, *lane 1*). As shown in Fig. 2, the upper bands at 41 and 40 kDa, which correspond to $G_{i_{1\alpha}}$ and $G_{i_{2\alpha}}$, respectively, were recognized by the antipeptide antibodies common to most of the α subunits (Fig. 2, *lane 3*) and by those specific for the carboxyl-terminal part of $G_{i_{1\alpha}}$ and $G_{i_{2\alpha}}$ subunits (Fig. 2, *lane 5*). As expected, the anti-$G_{o_{1,2\alpha}}$ antibody (raised against the purified $G_{o\alpha}$ subunit and recognizing two $G_{o\alpha}$ isoforms) labeled only the lower band at 39 kDa (Fig. 2, *lane 2*). Similarly, the affinity-purified anti-α_{o_1} and anti-α_{o_2} peptide antibodies (Fig. 2, *lanes 4* and *6*, respectively) recognized a single G_α subunit at 39 kDa, with identical electrophoretic mobilities. In this respect, it can be stressed that a cross-reactivity with $G_{i_{1\alpha}}$ or $G_{i_{3\alpha}}$ would have been easily detected with such resolving electrophoresis. It is also interesting to observe the weak labeling with the anti-$G_{o_{2\alpha}}$ antibody, which suggested a low level of this isoform in adult bovine brain (see below).

The specificity of the antipeptide antibodies was also evaluated by immunoblotting using the brain particulate fraction from 15-day-old mouse fetus, a tissue that contains a substantial level of $G_{o_{2\alpha}}$ isoform. The recognition patterns were compared (Fig. 3, *first lane of each panel*) with that obtained with the anti-$G_{o_{1,2\alpha}}$ antibodies used in our previous studies (11, 18, 19). Some additional bands were detected with the antipeptide antibodies, especially in the case of the $G_{o_{2\alpha}}$ antibodies, where there was labeling at about 35 kDa (Fig. 3, *right*). This labeling, not seen in adult animals, does not correspond to a $G_{o\alpha}$-like subunit, because there was no immunoreactivity with our common anti-$G_{o_{1,2\alpha}}$ antibodies at the same molecular weight. Nevertheless, the $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$ immunoreactivities could be specifically removed if the antipeptide antibodies were preincubated with their corresponding peptide antigens (Fig. 3, *lanes 5* and *9*, respectively). Interestingly, under similar conditions the $G_{o_{1,2\alpha}}$ immunoreactivity remained unaffected (Fig. 3, *lanes 2* and *3*). Thus, from the two experiments described in Figs. 2 and 3, the anti-α_{o_1} and anti-α_{o_2} peptide antibodies appear to

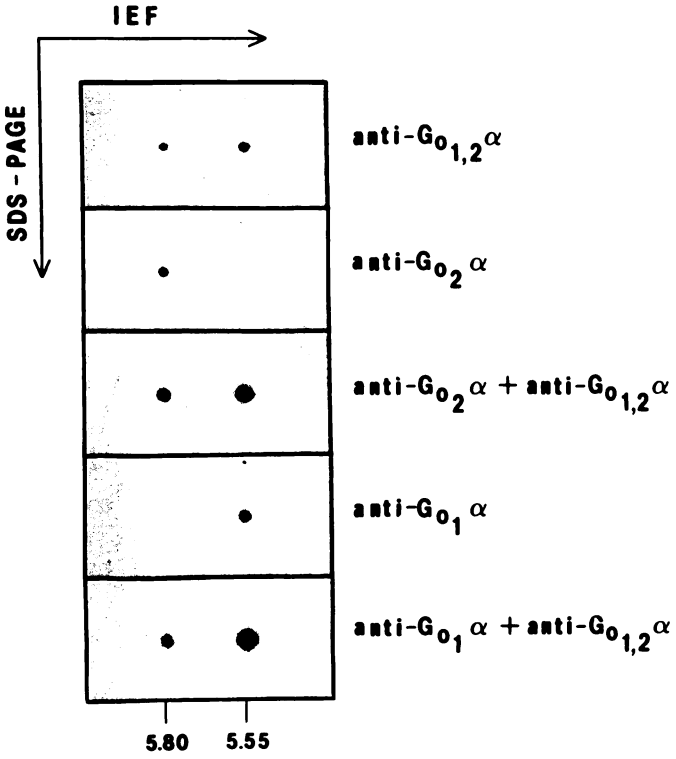

Fig. 5. Identification of $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$ protein in frontal cortex of mouse fetuses. Particulate fractions (50 μg of protein) of frontal cortex from 18-day-old fetuses were analyzed by two-dimensional electrophoresis and immunoblotting with affinity-purified anti-$G_{o_{1\alpha}}$ antibody (8 μg/ml), anti-$G_{o_{2\alpha}}$ antibody (13 μg/ml), or crude antiserum raised against purified bovine brain $G_{o\alpha}$ (anti-$G_{o_{1,2\alpha}}$, 1/200 dilution). Immunocomplexes were detected with ^{125}I-Protein A (200,000 cpm/ml). Autoradiographs were made from the blots with Kodak XAR 5 film after 48- or 72-hr exposure. Only the relevant portions of the autoradiograms are shown. When two different antibodies were used, treatment of blots was successive, first with specific antipeptide antibody and ^{125}I-Protein A and then, after exposure, with anti-$G_{o_{1,2\alpha}}$ followed by ^{125}I-Protein A. Only two isoforms of $G_{o\alpha}$ were detected on the autoradiograms after this procedure.

recognize specifically the polypeptide from which the antigen sequence was derived.

Identification of $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$ subunits in 72-hr differentiated neuroblastoma cells. We then assessed whether each antipeptide antibody could specifically recognize, after separation by two-dimensional gel electrophoresis, one of the two isoforms previously labeled by our common anti-$G_{o_{1,2\alpha}}$ antibodies. Fig. 4 reveals that anti-$G_{o_{1\alpha}}$ and anti-$G_{o_{2\alpha}}$ antibodies bound to a single spot with an apparent molecular mass of 39 kDa, focused at a pH of 5.55 and 5.80, respectively (Fig. 4, *first* and *third panels*). To confirm that these spots really correspond to $G_{o\alpha}$ isoforms, we reincubated the same two blots (Fig. 4, *first* and *third panels*) with our common anti-$G_{o_{1,2\alpha}}$ antiserum raised against the purified bovine $G_{o\alpha}$ protein. Under these conditions (Fig. 4, *second* and *fourth panels*), no additional spot could be detected, indicating that the proteins stained with the antipeptide antibodies have the same pI values as those recognized by the common anti-$G_{o\alpha}$ antibodies. Thus, $G_{o\alpha}$ isoforms focusing in two-dimensional gels at pH 5.55 and 5.80 possessed sequences similar to those of $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$ protein, respectively.

Identification of a $G_{o_{2\alpha}}$ protein in brains of mouse fetuses by two-dimensional gel analysis and immunoblotting. We have previously shown that only one isoform of $G_{o\alpha}$ protein, focused at a pH of 5.8, corresponding to $G_{o_{2\alpha}}$ protein, is expressed in undifferentiated neuroblastoma cells (11). Morphological differentiation of these cells appears to coincide with the expression of the more acidic $G_{o\alpha}$ isoform (pI 5.55), corresponding to $G_{o_{1\alpha}}$ protein. Furthermore, particulate fractions of primary culture of granule cells generated from cerebella of 7-day-old mice revealed the presence of only one $G_{o\alpha}$ isoform, focused at a pH of 5.55 (11). These observations suggest that $G_{o_{2\alpha}}$ might be expressed in neuronal cells before their differentiation; thus, we used brains from fetal mice in an attempt to detect a $G_{o_{2\alpha}}$ protein.

In particulate fractions from the frontal cortices of 18-day-old Swiss albino mouse fetuses, the antiserum raised against the purified bovine brain $G_{o\alpha}$ (anti-$G_{o_{1,2\alpha}}$) revealed two spots, which focused at pH 5.55 and 5.80 with about the same apparent molecular mass (39 kDa) after two-dimensional analysis and immunoblotting (Fig. 5, *first panel*). In contrast, the affinity-purified antibodies raised against the α_{o_2} peptide detected only one spot, migrating at 39 kDa with a pI of 5.80, suggesting that this spot is indeed $G_{o_{2\alpha}}$ (Fig. 5, *second panel*). To confirm this, we reincubated the same blot with the common anti-$G_{o_{1,2\alpha}}$ antibodies. Results indicated that the spot at pI 5.80 was increased, and only one new spot, at a pI of 5.55, appeared (Fig. 5, *third panel*). The affinity-purified antibody raised against the α_{o_1} peptide revealed only one spot, focused at a pI of 5.55 (Fig. 5, *fourth panel*). Incubation with the common anti-$G_{o_{1,2\alpha}}$ antibodies after anti-$G_{o_{1\alpha}}$ antibodies increased the spot at pI 5.55 (Fig. 5, *fifth panel*). Thus, this latter spot corresponds to $G_{o_{1\alpha}}$ protein. Particulate fractions derived from other parts of the brain of 18-day-old mouse fetuses (striata, colliculi, and cerebella) gave similar results but with a different ratio between the two $G_{o\alpha}$ isoforms (see below).

Differential expression of $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$ isoforms during brain development. Because we were able to detect both $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$ protein using two-dimensional gel analysis and immunoblotting with anti-$G_{o_{1,2\alpha}}$ antibodies, we used this approach to study the expression of these proteins during brain development. Fig. 6, *left*, shows autoradiograms after two-dimensional electrophoresis and immunoblotting, with anti-$G_{o_{1,2\alpha}}$ antiserum, of particulate fractions from frontal cortex of mouse fetuses and neonates. The two isoforms of $G_{o\alpha}$, $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$, focused as spots at pH 5.55 and 5.80, respectively. During development, the intensity of the spot corresponding to $G_{o_{2\alpha}}$ decreased and almost completely disappeared 30 days after birth (P30). In contrast, the intensity of the immunoreactive spot associated with $G_{o_{1\alpha}}$ increased, and only the $G_{o_{1\alpha}}$ isoform was detected at P30 (Fig. 6).

Fig. 6, *right*, is a quantitative analysis of the expression of the two $G_{o\alpha}$ isoforms, determined as described in Materials and Methods. This time course, obtained from particulate fractions of frontal cortex, indicates that $G_{o_{2\alpha}}$ protein decreases and disappears, whereas $G_{o_{1\alpha}}$ increases approximately 8-fold, between 15 days of gestation and 30 days after birth (Fig. 6). Note that the curves of $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$ expression intersected at 17 days of gestation. This period of time would correspond to a ratio of 1:1 between $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$ subunits if the isoforms are similarly recognized by the anti-$G_{o_{1,2\alpha}}$ antibody. This happens to be the case, as evaluated by comparison with antipeptide antibodies directed against common carboxyl- and amino-terminal sequences that are identical in both isoforms (data not shown). A similar time course was obtained with particulate fractions derived from striata, colliculi, and cerebella, but com-

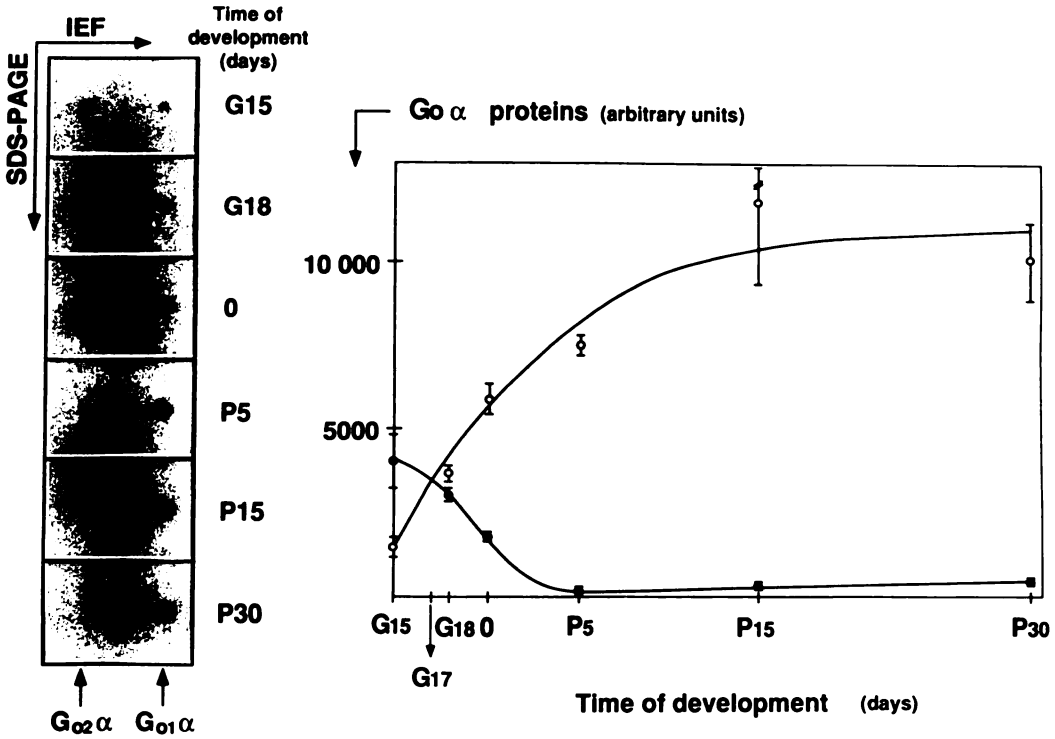

Fig. 6. Expression of $G_{o_1\alpha}$ and $G_{o_2\alpha}$ during frontal cortex development. Particulate fractions (50 μg) from frontal cortex of fetuses and newborns [after 15 and 18 days of gestation (*G15* and *G18*), at birth (*0*), and 5, 15, and 30 days after birth (*P5*, *P15*, and *P30*)] were subjected to two-dimensional gel analysis and immunoblotting with anti-$G_{o_{1,2}\alpha}$ antiserum, as described in Materials and Methods. *Left*, relevant portions of the autoradiograms derived from two-dimensional gel analysis and immunoblotting, showing spots corresponding to differential expression of $G_{o_2\alpha}$ and $G_{o_1\alpha}$ during development. Autoradiograms were made from the blots with Kodak XAR 5 film, after an exposure of 1 week without intensifying screens. *Right*, time course of expression of the two isoforms of $G_{o\alpha}$ proteins. Quantitative data were obtained by scanning the intensity of spots corresponding to the two isoforms of $G_{o\alpha}$ after SDS-PAGE and immunoblotting with anti-$G_{o_{1,2}\alpha}$ antiserum. The ratio between the two $G_{o\alpha}$ isoforms was obtained by two-dimensional gel analysis and immunoblotting with the same antiserum. Values are expressed as mean ± standard error of triplicate determinations.

parison of values of intersection between the expression curves of $G_{o_2\alpha}$ and $G_{o_1\alpha}$ indicated the following order: cerebella ≥ colliculi > striata > frontal cortex.

Differential expression of $G_{o_2\alpha}$ and $G_{o_1\alpha}$ during differentiation of neuroblastoma cells. As previously shown, differentiation involves the expression of two isoforms of $G_{o\alpha}$, which focused at pH 5.80 and 5.55 and possess peptide sequences derived from cDNAs of $G_{o_2\alpha}$ and $G_{o_1\alpha}$, respectively. In these clonal cells, differentiation is morphologically visualized by neurite outgrowth.

Two-dimensional analysis and immunoblotting with anti-$G_{o_{1,2}\alpha}$ antibodies indicated that nondifferentiated neuroblastoma cells possess only one isoform of $G_{o\alpha}$, which focused at a pH of 5.80 and corresponded to $G_{o_2\alpha}$. Differentiation involves the progressive expression of a more acidic isoform of $G_{o\alpha}$ (focusing at a pH of 5.55, corresponding to $G_{o_1\alpha}$) and a decline of the more basic $G_{o_2\alpha}$ protein (Fig. 7, *left*). The time course of the expression of both $G_{o_2\alpha}$ and $G_{o_1\alpha}$ proteins (Fig. 7, *right*) was constructed by scanning the autoradiograms from SDS-PAGE gels and two-dimensional gels that had been immunoblotted with anti-$G_{o_{1,2}\alpha}$ antiserum (see Materials and Methods). As shown in this figure, 120-hr differentiation was accompanied by a 30-fold increase in $G_{o_1\alpha}$ expression and a decline of 53 ± 5% (three experiments) in the expression of $G_{o_2\alpha}$

Discussion

The correspondence between the polypeptide sequence deduced from the two cDNAs now available and the various $G_{o\alpha}$ isoforms purified or identified after SDS-PAGE was not clearly established. The raising of specific antipeptide antibodies should provide a way to assess such correspondence unambiguously. However, between the two $G_{o\alpha}$ isoforms, only 26 amino acids are different and the longer sequence of "completely different consecutive amino acids" does not exceed six amino acids in length. Because peptides used to raise antibodies are generally composed of 10 or more amino acids, the α_{o_2} antigen will necessarily contain some amino acids identical to their homologues in the $G_{o_1\alpha}$ sequence. For this reason, it appeared that an unequivocal determination of $G_{o\alpha}$ isoforms would require the use, in parallel, of two specific antibodies directed against a homologue domain of the $G_{o_1\alpha}$ or $G_{o_2\alpha}$ polypeptides.

Among the pertussis toxin-sensitive G proteins purified from bovine brain, the affinity-purified antipeptide antibodies described in this study recognized only the $G_{o\alpha}$ isoforms (Fig. 2). Moreover, the presence of the peptide used as an antigen only blocked the recognition of the $G_{o\alpha}$ subunit by the antibody that was raised against that particular peptide. After separation by two-dimensional gel electrophoresis of proteins from particulate fractions of either differentiated neuroblastoma cells or mouse brain, only two spots with $G_{o_{1,2}\alpha}$ immunoreactivity were present. Each spot was recognized by only one of the antipeptide antibodies, and their specificity indicated that the isoform with a pI value of 5.80 includes in its amino acid sequence the α_{o_2} peptide, whereas that with a pI value of 5.55 is of the α_{o_1} type. Assuming that no differential post-translational modifi-

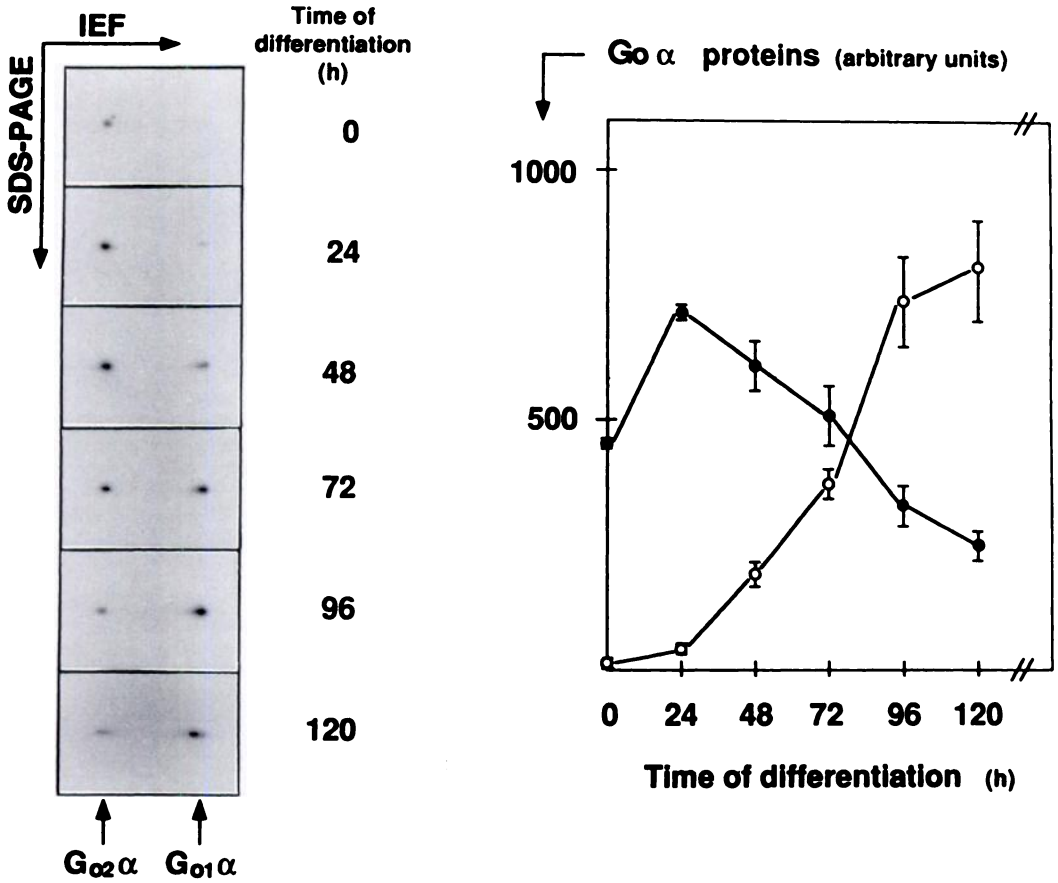

Fig. 7. Expression of $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$ during differentiation of N1E-115 neuroblastoma cells. N1E-115 neuroblastoma cells in the undifferentiated state (time 0) or at different stages of differentiation were homogenized, and particulate fractions were prepared as described in Materials and Methods. Particulate fractions (50 μg) of protein were subjected to SDS-PAGE and to two-dimensional analysis, followed by immunoblotting with anti-$G_{o_{1,2\alpha}}$ antiserum and ^{125}I-Protein A. *Left*, relevant portions of the autoradiograms from two-dimensional gel analysis and immunoblotting, showing spots corresponding to $G_{o_{2\alpha}}$ and $G_{o_{1\alpha}}$ during differentiation of N1E-115 neuroblastoma cells. Autoradiograms were exposed to Kodak XAR 5 film for 10 days without intensifying screens. *Right*, time course of expression of the two isoforms of $G_{o\alpha}$ proteins during N1E-115 cell differentiation. The ratio between the two $G_{o\alpha}$ isoforms was obtained by two-dimensional gel analysis and immunoblotting with anti-$G_{o_{1,2\alpha}}$ antiserum. The intensities of the G_{o_α} spots on autoradiograms were quantitated by densitometry in arbitrary units. Values are expressed as mean ± standard error of triplicate determinations.

cation occurs between the two $G_{o\alpha}$ isoforms, their relative pI values were expected, because the $G_{o_{2\alpha}}$ subunit is composed of four more basic residues than the $G_{o_{1\alpha}}$ protein. During the preparation of this manuscript, Spicher *et al.* (22), using an antibody analogous to our $G_{o_{2\alpha}}$ antibodies (their antigen peptide was 21-amino acids long and included our whole α_{o_2} peptide), also provided evidence for a positive α_{o_2} peptide immunoreactivity at 39 kDa in rat and hamster brain. At variance with our results in Fig. 2, these authors reported the absence of immunoreactivity in cholate extracts from porcine or bovine brain. We demonstrated here that, in brain from 15-day-old mice, the level of the $G_{o_{2\alpha}}$ subunit represented <5% (3.6 ± 0.4%; three experiments) of the total $G_{o\alpha}$ immunoreactivity. Probably the nonrodent animals used in Spicher's experiments were older than the rodent species and thus possess a level of $G_{o_{2\alpha}}$ subunit too low to be immunologically detected under their conditions.

The discrimination of two isoforms has also been reported using one-dimensional SDS-urea-PAGE with 4–8 M urea (12, 21–23). Although the separation between the two main isoforms is less than with two-dimensional gels, the two $G_{o\alpha}$ isoforms described here can also be identified. Thus, Granneman and Kapatos (24) showed, in primary culture of hippocampal neurons, that between day 4 and day 14 after plating the isoform of higher mobility disappeared from SDS-urea-PAGE, whereas the isoform just above it (lower mobility) was increased. Comparison of this observation with our immunological results and those from others (12, 22–24) indicates that the isoform of higher mobility on SDS-urea-PAGE is of the $G_{o_{2\alpha}}$ type, whereas the isoform just above it is indeed of the $G_{o_{1\alpha}}$ subtype. Sometimes, using this kind of gel, a third form of $G_{o\alpha}$ with a slower electrophoretic mobility, which might be the α_o* subunit previously purified (8), was also identified. One plausible explanation for the absence of this latter form in our two-dimensional gel could be partial solubilization of the protein during membrane preparation, as observed previously (24), or the protein might be present at a level too low to be identified. Alternatively, it is possible that this isoform could not penetrate the isofocusing gel. Whatever the reasons for the absence of this isoform from the two-dimensional electrophoresis, it remains to be determined immunologically whether this slowly migrating α_o subunit is of the α_{o_1} or α_{o_2} subtype.

The use of specific antibodies has not only permitted the identification of the two isoforms but has also provided evidence for an on/off switch between the $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$ subunits

during cell differentiation. Thus, cell lines of neuronal origin (N1E-115, NG108-15) and mouse or rat brain during the early stage of development mainly express the $G_{o_{2\alpha}}$ subunit, whereas the predominance of $G_{o_{1\alpha}}$ occurs only after neuronal differentiation. Because the rate of degradation is equivalent for both $G_{o_{1\alpha}}$ and $G_{o_{2\alpha}}$ isoforms (25), it seems that differentiation turns on the alternative splicing. Although the precise mechanism of the on/off switch remains to be clarified, the antipeptide antibodies, the specificities of which have been characterized in this work, will also be of use in determining precisely the tissue and cellular localization of each $G_{o\alpha}$ isoform, especially in peripheral tissues where mRNAs of both isoforms have already been detected (14, 15).

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