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Ras protein expression is developmentally regulated in embryonic chicken brain

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Immunoblot analysis using a panreactive monoclonal antibody directed against ras p21 proteins detects two differentially regulated ras pools in embryonic chicken brain: a membrane pool that is not changed and a microsomal pool that starts at a low level but is strongly increased from E6 to E16 chicken brain. In order to study the distribution of ras proteins in different cell types of the nervous system, immunoblot analysis was performed on total cell proteins. In contrast to histochemical data showing the absence or low levels of ras proteins in glial cells, comparable amounts of ras proteins were found in cell lysates from purified chicken sympathetic neurons, cultured rat Schwann cells and mouse brain astrocytes.

Ras p21 protein; Immunoblot analysis; Membrane pool; Microsomal pool; Developmental regulation; Rodent glial cell; (Embryonic chicken brain)

1. INTRODUCTION

The ras family of oncogenes was discovered in Harvey and Kirsten murine sarcoma viruses and was subsequently shown to exist in vertebrate cells as protooncogenes. Members of the mammalian ras protooncogene family, c-Ha-ras, c-Ki-ras and N-ras, each encode guanine nucleotide binding proteins of 21 kDa (p21 proteins) which are associated with the inner side of the plasma membrane (for review see [1]). Sequence homology between ras proteins and α-subunits of G-proteins involved in signal transduction has led to the proposition that these proteins have analogous functions [2]. Mammalian ras genes acquire transformation-inducing properties by single point mutations within their coding sequences (for review see [1]). The biological function of ras proteins in mammalian cells is poorly understood. It was shown that ras protein function is required for serum-stimulated growth of NIH3T3 cells [3]. In addition to their transforming properties, microinjected ras proteins also induce terminal differentiation of PC12 cells [4] and promote survival and fiber outgrowth of cultured embryonic neurons [5]. Besides immature cells capable of proliferation, fully differentiated cells like neurons express abundant ras p21 [6]. Among mammalian tissues the highest level of ras proteins was detected in brain [6,7] and subsequently shown to be localized mainly in synaptic plasma membranes and microsomes [8]. This study demonstrates the presence of two ras pools in chicken brain which are differentially regulated during embryonic development.

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Abbreviations: E6, embryonic day 6; GFAP, glial fibrillary acidic protein; NF68, neurofilament 68; mAb, monoclonal antibody; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

Chicken brains from different embryonic stages were subjected to Dounce homogenization and membranes (25000 × *g* fraction) were prepared as described elsewhere [9]. Microsomes (200000 × *g* fraction) were isolated by spinning the 25000 × *g* supernatant for 1 h at 200000 × *g*. Proteins were assayed by the method of Peterson [10] using bovine serum albumin as a standard. The isolation and purification of chicken sympathetic neurons [11], rat Schwann cells [12] and mouse brain astrocytes [13] was done as described. Cell lysates were prepared by boiling the cells in sample buffer. Proteins together with Rainbow protein molecular weight markers (Amersham Buchler, Braunschweig, FRG) were separated on 12% SDS-polyacrylamide gels prepared by the method of Laemmli [14] and then electrotransferred onto nitrocellulose sheets (BA83, 0.2 μm; Schleicher & Schuell, Dassel, FRG) for 15 h at 100 V [15]. The nitrocellulose sheets were then reacted with the appropriate dilution of mouse monoclonal antibody followed by anti-mouse IgG alkaline phosphatase conjugate as specified by the manufacturer's instructions (Promega, Madison, WI, USA). The following monoclonal antibodies were used in this study: anti-ras p21 (TuMark-Ras 11; NEN, Dreieich, FRG), anti-NF68 (clone NR-4; Sigma, Deisenhofen, FRG) and anti-GFAP (clone G-A-5; Sigma). Staining of total proteins on nitrocellulose blots was performed with FerriDye (Janssen, Beerse, Belgium).

3. RESULTS AND DISCUSSION

In contrast to most other mammalian tissues, brain contains high levels of ras p21 proteins [6–8]. Using a panreactive monoclonal antibody directed against ras

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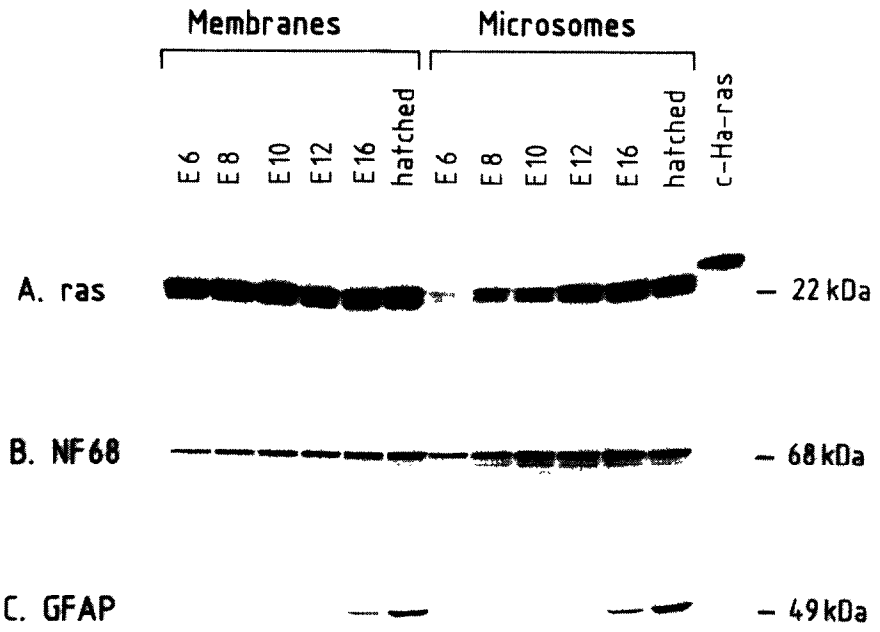

Fig.1. Immunoblot analysis of membrane and microsomal proteins prepared from different days of embryonic chicken brain with antibodies specific for ras (A), NF68 (B) and GFAP (C). 50 μg of membrane or microsomal proteins for ras and GFAP and 20 μg for NF68 were subjected to SDS-PAGE, transferred to nitrocellulose and reacted with monoclonal antibodies against ras (1:1000), NF68 (1:5000) or GFAP (1:5000). Right lane in A: 5 ng of recombinant c-Ha-ras protein. Values on the right indicate the apparent molecular masses (kDa).

proteins [16], I find by immunoblot analysis high amounts of ras proteins in membrane (25000 × *g*) and microsomal (200000 × *g*) fractions prepared from embryonic chicken brain. Fig.1A shows that this antibody recognizes two ras species with apparent molecular masses of 22 and 23 kDa in membranes and microsomes from chicken brain. Also in subcellular fractionation analysis of rat cerebrum ras proteins were found mostly in synaptic plasma membranes and microsomes [8]. I further used recombinant c-Ha-ras protein [17] to demonstrate the specificity and sensitivity of this antibody. The reaction conditions used here are sensitive enough to detect 5 ng of recombinant c-Ha-ras protein with an apparent molecular mass of 24 kDa (fig.1A, right lane). The molecular weight of recombinant ras proteins is higher than that of the cellular counterparts due to lack of posttranslational processing of ras proteins produced in *E. coli* [18–20]. When compared to the recombinant c-Ha-ras standard, ras proteins in membrane and microsomal fractions from hatched chicken brain represent about 0.02% of total protein in these fractions. Since no sensitive antibodies specific for the subtypes of ras proteins are available at the moment, I cannot discriminate whether the ras doublet found in chicken brain is caused by the presence of two subtypes or by different posttranslational modification of one predominant subtype.

Using this sensitive immunoblot system, I looked for developmental changes in expression of ras proteins in embryonic chicken brain. As can be seen in fig.1A, ras proteins are present at high levels in membranes prepared from embryonic day 6 (E6) to hatched chicken brain. In contrast, there is a strong increase in content of ras proteins in microsomal fractions from chicken brain between E6 and E16. Whereas at E6 about 10-fold more ras proteins are present in membranes compared to microsomes, no difference can be seen at E16 and hatched chicken brain. Apparently there are two pools of ras proteins in embryonic chicken brain which are differentially regulated in development. Staining of transferred membrane and microsomal proteins on nitrocellulose blots demonstrated the presence of comparable amounts of proteins from different developmental stages (data not shown). Interestingly I found a change in the proportion of membranes and microsomes during chicken brain embryonic development. Whereas at E6 membrane proteins (35%) constitute a smaller portion of the particular fraction than microsomal proteins (65%), in hatched chicken brain the situation is reversed. The decrease in microsomes cannot explain the strong increase of ras proteins in this fraction.

I further used monoclonal antibodies against the neuronal cell marker protein neurofilament 68 (NF68) and the glial cell marker protein glial fibrillary acidic protein (GFAP) [21] to study the developmental pat-

tern of these proteins in membranes and microsomes from embryonic chicken brain. Fig.1B shows that anti-NF68 detects a protein with an apparent molecular mass of 68 kDa in the particulate fractions. In membranes there is a pronounced increase in NF68 levels which starts at E6 and continues up to hatched chicken brain, whereas in the microsomal fraction maximal levels of NF68 are reached at E16. As can be seen in fig.1C, anti-GFAP recognizes a protein with an apparent molecular mass of 49 kDa on immunoblots of particulate fractions derived from embryonic chicken brain. GFAP cannot be demonstrated before E12 and is strongly increased up to hatched chicken brain. The appearance of NF68 precedes that of GFAP in developing chicken brain, which is in accordance with a previous immunofluorescence study in the rat embryo [22]. By comparison of developmental changes in ras proteins, NF68 and GFA, it becomes clear that levels of NF68 and GFAP increase in a similar fashion in membranes and microsomes from developing chicken brain, whereas the expression of ras proteins is differentially regulated in these two fractions. The underlying mechanism which causes the differential expression of ras proteins in membranes and microsomes from developing chicken brain and its biological meaning remains to be elucidated. The finding that ras proteins and NF68 show a similar increase in the microsomal fractions may suggest that the microsomal ras pool increases in parallel with the neuronal characteristics of developing chicken brain.

There is evidence that ras proteins are involved in the intracellular signal transduction pathway for neurotrophic factors in neuronal cells [4,5]. In the particulate fraction of embryonic chicken brain I find an increase in the level of ras proteins from E6 to E16. This increase in amount of ras proteins is opposed to the decrease in NGF receptor levels found in regions of chicken brain between E6 and E12 [23]. Consequently the expression of ras proteins and NGF receptors is not coregulated during chicken brain embryonic development.

The immunohistochemical staining of ras proteins in mammalian brain by monoclonal antibodies has led to controversial results. Using the mAb Y13-259, Furth et al. found strong staining of neurons in human central and peripheral nervous system, whereas glial cells and astrocytes were weak or negative [6]. In contrast, Ward et al. [24] could find no ras immunoreactivity in rodent brain tissue using several mAbs against ras proteins including Y13-259.

To get some insight into the distribution of ras proteins in cells of the nervous system, I analyzed by immunoblotting the presence of ras proteins in cell lysates from purified chicken sympathetic neurons, rat Schwann cells and astrocytes from mouse brain. These cells were isolated and purified as described [11–13] and were lysed by boiling in sample buffer. As can be seen in fig.2, immunoreactive bands of the expected molecular weight are present in chicken neurons, rat Schwann cells and mouse astrocytes. For comparison the pattern of ras proteins was analyzed in membranes prepared from chicken, rat and mouse brain. Fig.2 and other unpublished results point to a species-specific pattern of ras proteins. Whereas chicken brain contains two ras species with apparent molecular masses of 22 and 23 kDa, rat and mouse brain contain two predominant ras species of 21 and 23 kDa. Interestingly, the ras patterns of purified cells are different from that of brain membranes. In contrast to chicken brain, in sympathetic neurons the 22 kDa form is more abundant than the 23 kDa form. In rat Schwann cells and mouse brain astrocytes the 23 kDa form is expressed whereas the 21 kDa form present in rat and mouse brain cannot be detected. These results provide clear evidence that ras proteins are present in astrocytes and Schwann cells at levels comparable to that of neurons. Further evidence for the presence of ras proteins in astroglial cells comes from the analysis of membranes derived from several mammalian glial cell lines (unpublished data). I cannot, however, exclude the possibility that expression of ras proteins in primary astroglial cells and glial cell lines is induced as a result of cell culture. The demonstration of NGF receptors on glial cells [25] might be in favour of the finding that ras proteins are clearly detectable in these cells. It is unlikely that one of the major bands in fig.2 represents an immature form of ras proteins, since Mizoguchi et al. [8] found that in rat brain ras proteins are almost exclusively

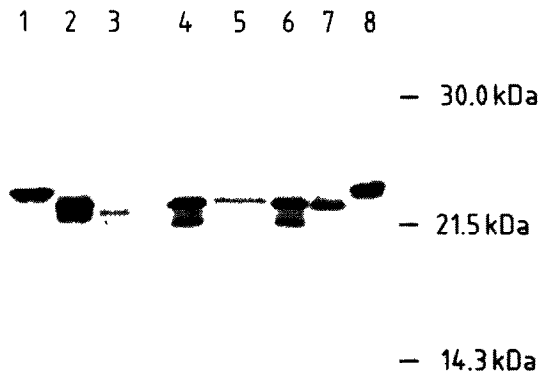

Fig.2. Immunoblot analysis of membrane proteins from brain and proteins from cell lysates with a panreactive monoclonal antibody against ras proteins. Recombinant c-Ha-ras protein (lanes 1 and 8), membrane proteins (lanes 2, 4 and 6) and comparable amounts of cell lysate proteins (lanes 3, 5 and 7) were subjected to SDS-PAGE, transferred to nitrocellulose and reacted with anti-ras mAb (1 : 1000). (Lane 1) 5 ng recombinant c-Ha-ras protein. (Lane 2) 40 μg membrane proteins from E12 chicken brain. (Lane 3) Cell lysate from E12 chicken sympathetic neurons. (Lane 4) 20 μg membrane proteins from rat brain. (Lane 5) Cell lysate from rat Schwann cells. (Lane 6) 20 μg membrane proteins from mouse brain. (Lane 7) Cell lysate from mouse brain astrocytes. (Lane 8) 2 ng of recombinant c-Ha-ras protein. Values on the right indicate the position of molecular mass marker proteins (kDa).

associated with the membrane and microsomal fractions and not with the cytosol. Furthermore immature ras proteins detected in the cytosol of ras-transformed cells have a lower electrophoretic mobility than their mature counterparts [26]. Due to the lack of sensitive antibodies against the subtypes of the ras protein family, I have no information on what subtypes are expressed in these cells. On the other hand, my results indicate that different subtypes and/or posttranslationally modified forms of ras proteins are expressed differentially in cells of the nervous system. The biological significance of this finding is unclear at the moment, but suggests a cell-specific function for different ras species.

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