

Immunocytochemical distribution of *raf* protein kinases and protein pattern in rat cerebellum

Jeong Soon Park and Won Chul Choi[†]

Department of Biology, College of Natural Sciences, Pusan National University, Pusan, 609-735, Korea

Abstract

a- and c-*raf* protein kinases in the brain of rat, the protein patterns of cerebellum during postnatal development of rat by polyacrylamide gel electrophoresis, and the existence of c-*raf* protein kinase by using Western blotting method.

The results were as follows :

The cytoplasm of Purkinje cells was, in general, strongly labeled with the antibodies of a- and c-*raf* protein kinases in the cortex regions such as *Pyramis cerebelli*, *Uvula*, *Nodulus*, *Paraflocculus*, and *Flocculus*. C-*raf* protein kinase appeared stronger immunoreactivity than a-*raf* protein kinase. In peripheral of cytoplasm of *Nucleus emboliformis*, A-*raf* Protein kinase was labeled markedly.

During postnatal development, the protein of 38,000 dalton increased gradually in the cytosolic fraction of cerebellum, and the protein of 260,600 dalton appeared in the membrane fraction of cerebellum. By immunoblotting method, the protein band of 74,000 dalton was detected in crude and cytosolic fractions, but it was not exhibited in membrane fraction.

In this fact, it was identified that a- and c-*raf* proteins were distributed throughout neuronal cells, especially in the Purkinje cells, in normal cerebellum cortex of rat. Also, this phenomenon was assumed that *raf* protein kinase in cytoplasm of neuronal cell had to do with a certain functional mechanism and signal transduction of neurotransmitter as Protein kinase C.

Key words : a- and c-*raf* protein kinases, Purkinje cells, cytosolic fraction, membrane fraction, immunoblotting, immunocytochemistry.

Introduction

More than 40 oncogenes has been identified by retroviral transduction and NIH 3T3 cell transfection assay¹⁾. It was discovered that *raf* gene encodes protein which has a serine/threonine-specific protein kinase activity^{2,3)}. V-*raf* was transformed fibroblast and epithelial cells in Murine Sarcoma Virus, induced fibrosarcoma in

new born mice⁴⁾. Also, it was ascertain that the nucleotide sequence of *raf* is different from other oncogenes. C-*raf* in cellular counterpart of v-*raf*⁵⁾, was identified in transformants induced by a human gastric cancer and glioblastoma lines^{6,7)}.

Recently, a-*raf* was discovered apparently to distinguish with c-*raf*, and closely related to other cellular gene^{8,9,10)}. A-*raf* appeared to associate with serine/threo-

[†] Corresponding author

nine-specific protein kinase activity similar to *c-raf*¹¹⁾, and *a-raf* gene contained cystein-rich regions in amino-terminal which seems likely to found in Protein Kinase C (PKC)^{12,13,14,15)}.

PKC have a similar with *raf* amino acid sequence, was widely found in many tissues of animal kingdom^{16,17,18,19)}. And PKC was associated with phosphorylation into neuronal function. It was reported that the distribution of PKC in brain studied using antibody against PKC^{20,21,22)}. PKC was strongly exhibited in the Purkinje cells of cerebellum of rat, but was scarcely shown in molecular cell layer and granular cell layer²³⁾.

This report describes the protein pattern during the postnatal development and the distribution of *raf* protein kinase in itelic rat cerebellum.

MATERIALS and METHODS

The cerebellum of rat isolated after it was perfused on phosphate buffered saline(pH 7.4). For electrophoresis, crude is prepared as follows: cerebellum was homogenized in sample extraction buffer(pH 7.4; 10mM Tris/Hcl, 5mM EGTA, 0.1mM DTT, 2mM PMSF), and then centrifuged in 14,000×g for 30 min., and supernatant was suspended. And supernatant was ultrasonicated, centrifuged in 100,000×g, cytosolic fraction is its supernatant. Membrane fraction is prepared that was centrifuged in 29,000×g after the pellet was suspended on sample buffer containing 0.5% Sodium Deoxycholate, and then ultrasonicated. Protein assay was according to Lowry's method²³⁾.

For SDS/polyacrylamide gel electrophoresis, gel was prepared with 6-16% linear gradient, buffer system was according to Laemmli's method²⁴⁾. Bands was scanned in the 1.0 density range after it stained 0.2% Coomassie brilliant blue R-250 solution. The analysis of molecular weights depend on Choi's computer program²⁵⁾.

The cerebellum was removed and fixed in the periodate-lysine-phosphate solution(PLP; pH7.4, 2% (wt/

vol) formaldehyde, 1.5% lysine, and 0.2% sodium-m-periodate, sodium phosphate)²⁶⁾ for 24hr. Cryo-cut sections of the cerebellum collected into 0.1M potassium phosphate buffer saline (KPBS, pH7.4). By Wood's method²⁷⁾, the sections were first blocked by incubating for 1hr into normal goat serum, and following incubated with primary antiserum in KPBS(diluted from 1:1000 and containing 0.1% Triton X-100) for 48hrs at 4°C and then washed three times in KPBS for 5 min.

The tissue was then sequentially incubated for 2hr with biotinylated goat anti-mouse IgG in room temperature. After washing, the section incubated for 1hr in the streptavidin conjugated horse raddish peroxidase. Then the sections was washed two times with 0.2M sodium acetate buffer (pH6.0), and stained with 0.1M sodium acetate buffer, pH6.0 containing 5mg of 3,3' diaminobenzidine, 20mM dextran, 7.5mM NH₄Cl, and 25 units of glucose oxidase for 30min at roon temperature. After staining, the sections were fixed for 10 min in 10% fromaline and then mount with gelatin coated slide.

For immunoblotting of *raf* protein, isolated cytosol and membrane fraction were sonicated and 120μl of protein were subjected to SDS-PAGE and immunoblotting using antibodies have been shown to be specific to a- and *c-raf* protein kinase. After immunoblotting, the nitrocellulose sheet was incubated for 1hr in 2% BSA in PBS to block nonspecific binding sites, and then washed with 0.05% Tween-20 in PBS. Following 24hrs incubation with the primary antibody (diluted 1:1000 in PBS containing 1% BSA), and was washed 3 times with PBS for 10min.

The membrane was incubated in antimouse IgG for 4 hrs and with peroxidase-conjugated rabbit anti-peroxidase antigene antibody complex for 2hrs at room temperature. The nitrocellulose sheet was washed with PBS containing 0.05% Tween-20.

Immunoreactive bands were visualized by treatment with 4-chloro-1-naphthol and hydrogen peroxide(1:1).

RESULTS

The distribution of *raf* protein kinase into cerebellum of rat was sectioned through the middle part of cerebellum for observing immunocytochemically. The region of cerebellum was shown in *Pyramis cerebelli*, *Uvula*, *Nodulus*, *Paraflocculus*, *Flocculus*, and *Nucleus emboliformis* was labelled with antibodies against *a-raf* and *c-raf*. The molecular layer of *Pyramis cerebelli* consisted of the dendrites of neuronal cells, was scattered about basket cells. However, to treat with *a-raf* antibody, the dendrites of neuronal cell was not labelled (Fig. 1). But Purkinje cells are between molecular layer and granular layer, was labelled. All Purkinje cell was not labelled with antibody against *a-raf*. *a-raf* protein kinase was not appeared in basket cells of molecular layer. *c-raf* was not labelled in the dendrites or basket cells of molecular layer as like to *a-raf*. But in the Purkinje cell, the antibody against *c-raf* was higher than *a-raf* in immunoreactivity. *c-raf* protein kinase was labelled in the cell body of Purkinje cells, and the fasciculated dendrites of cells was not labelled in *c-raf* (Fig. 2). And, in the granular layer of *Pyramis cerebelli*, antibodies of *a-* and *c-raf* was not labelled in granule cell, golgi cell, basket cell, and satellite cell. It was stained a similar with the constituent substances of these tissues, but did not apparently appear as the purkinje cells (Fig. 1, 2).

In the molecular layer of *Uvula*, the antibody against *a-raf* was shown lower than *c-raf* in immunoreactivity, the neuronal cells consist of this layer appeared markedly than other region. The Purkinje cell was labeled with *a-raf* protein kinase, but all was not labeled (Fig. 3). And it was interesting that the labeling pattern of the Purkinje cells was shown in the peripheral of cytoplasm as like ring-form. However, *c-raf* protein kinase appeared most evident in the Purkinje cell than *a-raf* (Fig. 4). In the granular layer, it labeled more obvious than the cell of molecular layer (Figs. 3, 4). But in this region, sometimes, it is observed that the cells like as pear-like cell

was labeled with *c-raf* (Fig. 4).

In the molecular layer of *Nodulus*, the cells of this region was not labeled almost with *a-* or *c-raf* Protein kinase, the Purkinje cell was higher immunoreactivity against *a-raf* than others (Fig. 5). The cell body of the Purkinje cells was labeled generally, but in the nucleus of cells, it was not reacted entirely to the *c-raf* protein kinase, it was strongly labeled in the cytoplasm (Fig. 6). Therefore, its appearance was shown as like gablet. In the granular layer of the *Nodulus*, granule cells was immunoreacted stronger with *a-raf* than *c-raf*, but in the Purkinje cells, appeared higher *c-raf* than *a-raf*.

In the *Paraflocculus* and *Flocculus*, the molecular layer of two region was not reacted to *a-*, *c-raf* protein kinase, but the Purkinje cell appeared stronger immunoreactivity the antibody against *a-raf* than *c-raf*. However, the pattern of immunoreactivity against these appeared a similar with other region of the cerebellum as above (Figs. 7, 9). And in granular layer, even through granule cells was lower reacted to antibodies than in Purkinje cell, it appeared that this layer was reacted to the antibodies against *a-raf* or *c-raf* (Figs. 7-9).

In *Nucleus emboliformis*, the middle size of cell was labeled with *a-raf* (Fig. 11), the larger cell than these cell was labeled with *c-raf* (Fig. 12). The shape of cell that labeled with *c-raf* was a circular conic form, often, the volume of nucleus in the cell is large. Therefore the spherical and transparent nucleus in the middle of a circular conic cells was observed (Figs. 11, 12).

As mentioned above, the molecular layer that is the outside of cortex of cerebellum, and granular layer that is the inside of cortex of cerebellum, were labeled lower with *a-* and *c-raf* than the purkinje cell layer between these layers. However, in granular layer, *c-raf* protein kinase was shown stronger immunoreactivity than *a-raf* protein kinase.

In the postnatal development of rat, the protein pattern of the cerebellum was compared with crude, cytosolic, and membrane fraction that was distinguished

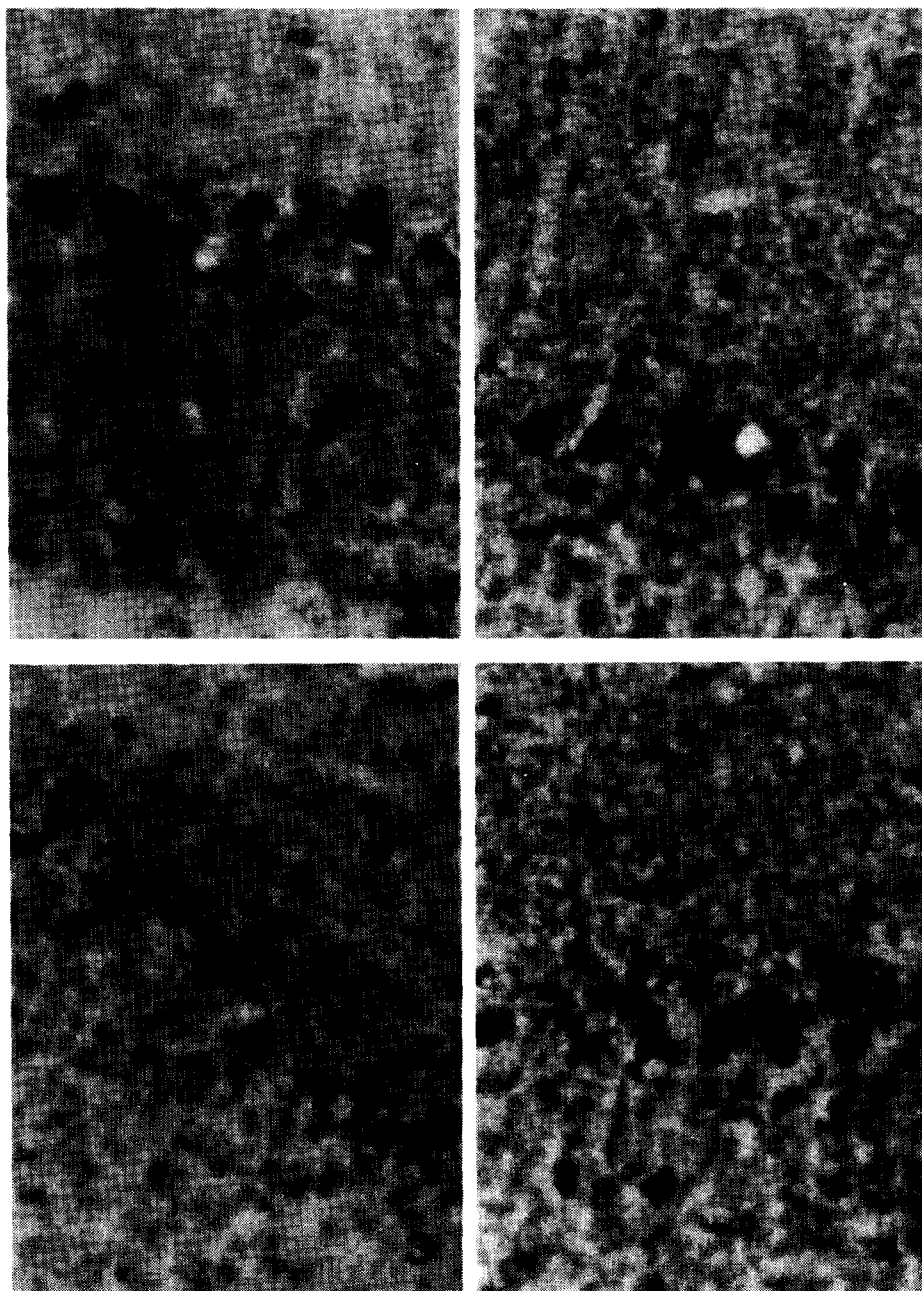


Fig. 1. Distribution of *a-raf* protein kinase in *Pyramis cerebelli*.

Fig. 2. *C-raf* protein kinase is occurred little lower immunoreactivity than *a-raf* protein kinase in *Pyramis cerebelli*.

Fig. 3. In *Uvula*, the cytoplasm of purkinje cells are occurred immunoreactivity against *a-raf* protein kinase.

Fig. 4. *C-raf* protein kinase is stronger immunolabeled than *a-raf* protein kinase in *Uvula*.

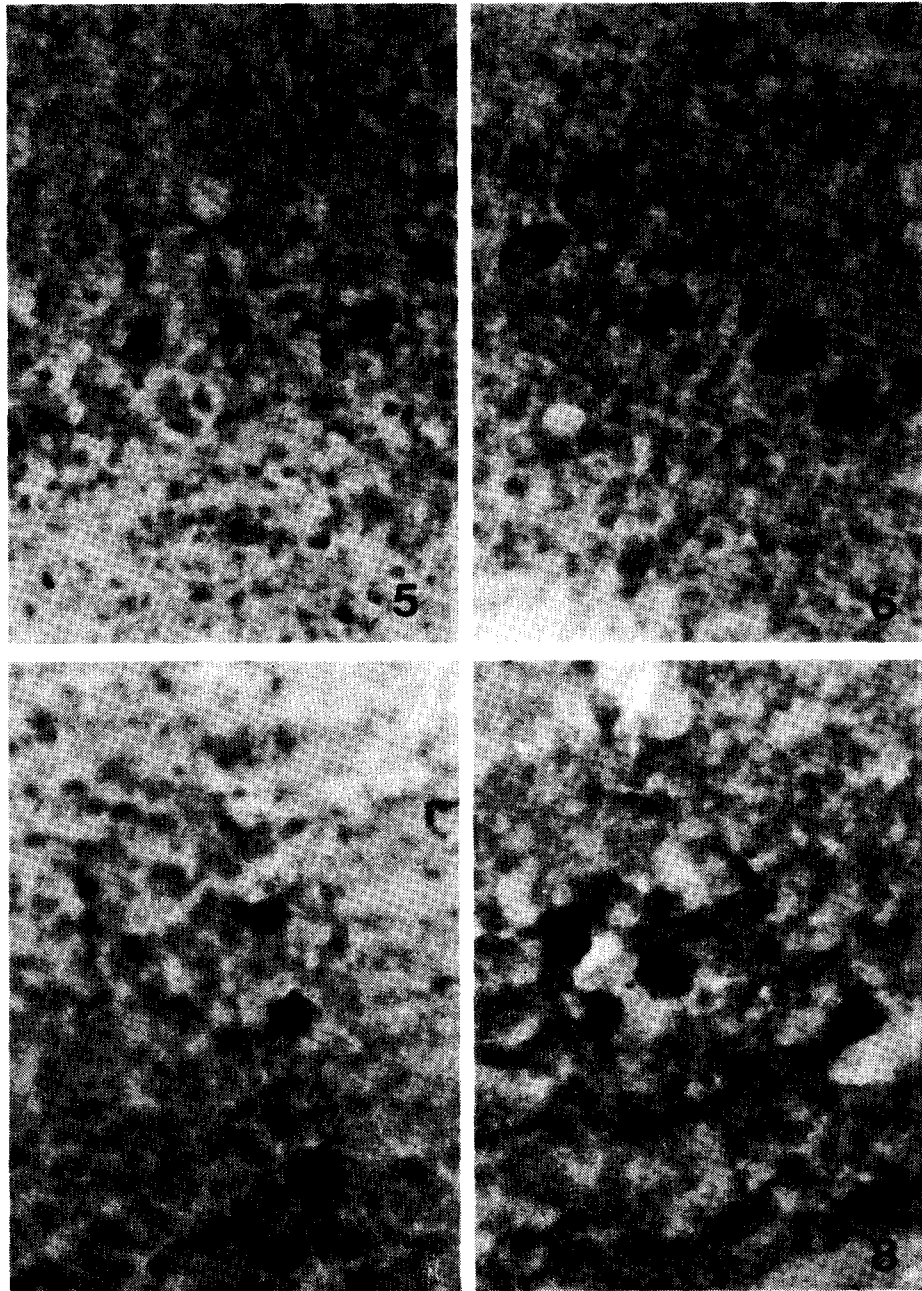


Fig. 5. Purkinje cells and granular cells are stained against *a-raf* protein kinase antibody in *Nodulus*.

Fig. 6. In *Nodulus*, *c-raf* protein kinase is occurred little lower immunoreactivity than *a-raf* protein kinase.

Fig. 7. The neuronal cells in *Paraflocculus* are densely labeled with *a-raf* protein kinase antibody.

Fig. 8. *C-raf* protein kinase are stronger stained than *a-raf* protein kinase in *Paraflocculus*.

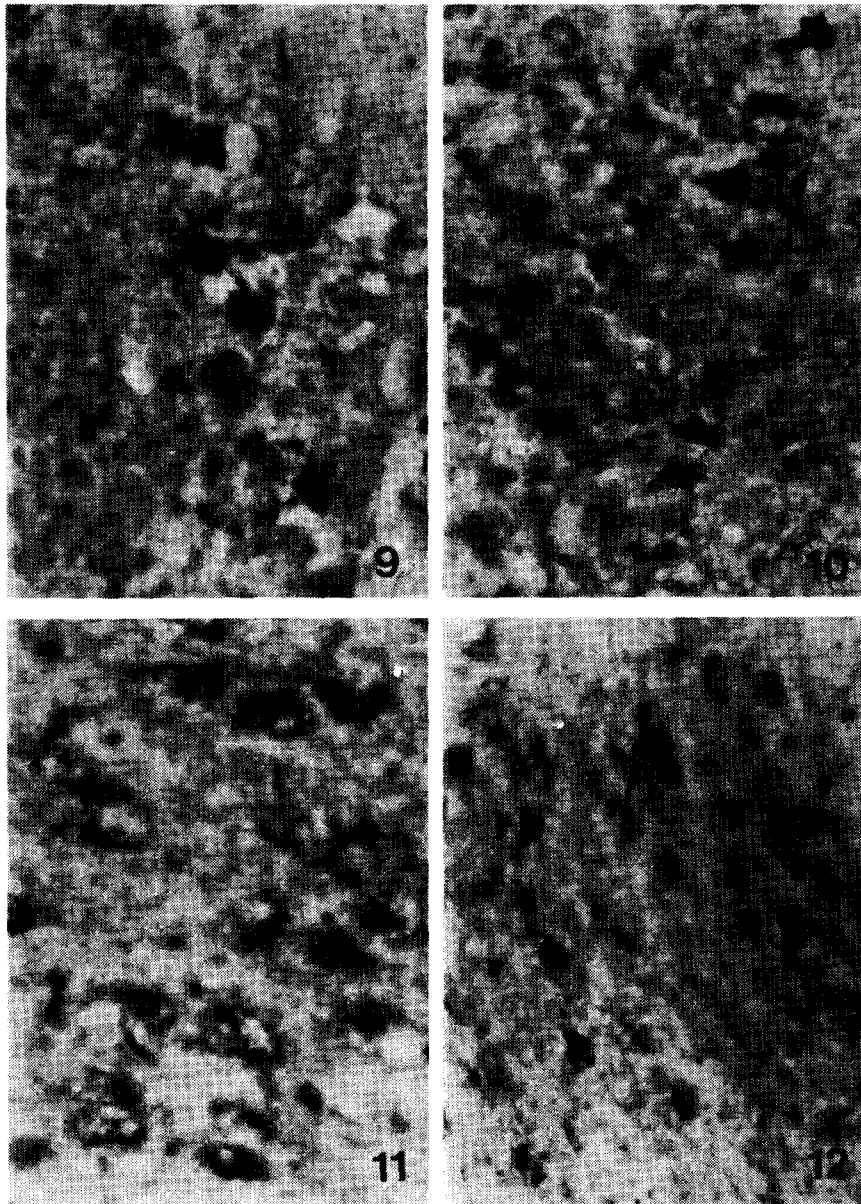


Fig. 9. Cytoplasm of neuronal cells is reacted with *a-raf* protein kinase antibody in *Flocculus*.

Fig. 10. In *Flocculus*, cytoplasm of Purkinje cell is labeled with *c-raf* protein kinase antibody.

Fig. 11. Peripheral region of neuronal cells in *Nucleus emboliformis* is densely labeled with *a-raf* protein kinase antibody.

Fig. 12. In *Nucleus emboliformis*, cytoplasm of neuronal cells is densely labeled with *c-raf* protein kinase antibody as like fig. 11.

Table 1. Molecular weights of protein of cerebellum

Bands	M.W.(dalton)	Bands	M.W.(dalton)
1	14,600	10	39,700
2	15,600	11	43,200
3	17,100	12	44,200
4	25,700	13	49,200
5	26,900	14	63,800
6	30,600	15	82,800
7	32,600	16	98,400
8	34,100	17	169,000
9	38,000	18	260,600

into the 5-old-day, 35-old-day, 90-old-day. The molecular weights of the protein of crude are shown in Table 1.

In the cytosolic fraction of 5-old-day cerebellum (Fig. 13 B), the bands 1,2,3, (M.W. 17,100-14,600 dalton) appeared much amounts, while in the membrane fraction (Fig. 13 C), these bands appeared few, and the protein amounts of the bands appeared a few and the protein amounts of the band 9(38,000) was few in cytosolic fraction. while the band 11 (43,200) appeared a good deal of amount in the membrane fraction (Fig. 13 C). In the membrane fraction, the protein amount of band 18(260,600), was shown few, but it was not shown in the cytosolic fraction (Fig. 13 B).

In the protein pattern of the 35-old-day cerebellum (Fig. 14), the amount of protein band 2(15,6000) was less than the 5-old-day cerebellum, the amount of protein of bands 10(39,700) and 11 appeared almost in large quantities. And the band 7 that did not appeared at the 5-old-day cerebellum was shown few in the 35-old-day. It appeared that the protein of band 2 was in the cytosolic fraction (Fig. 14 B), the band 6, 7, and 8 (30,600, 32,600, and 34,100) appeared abundant. And the protein of band 9 appeared in large quantities in the cytosolic fraction, but the protein of band 18 in membrane fraction is almost the same as the protein pattern of 5-old-day (Fig. 14 C). It means that the proteins of bands 2 and 9 in the cytosolic fraction was es-

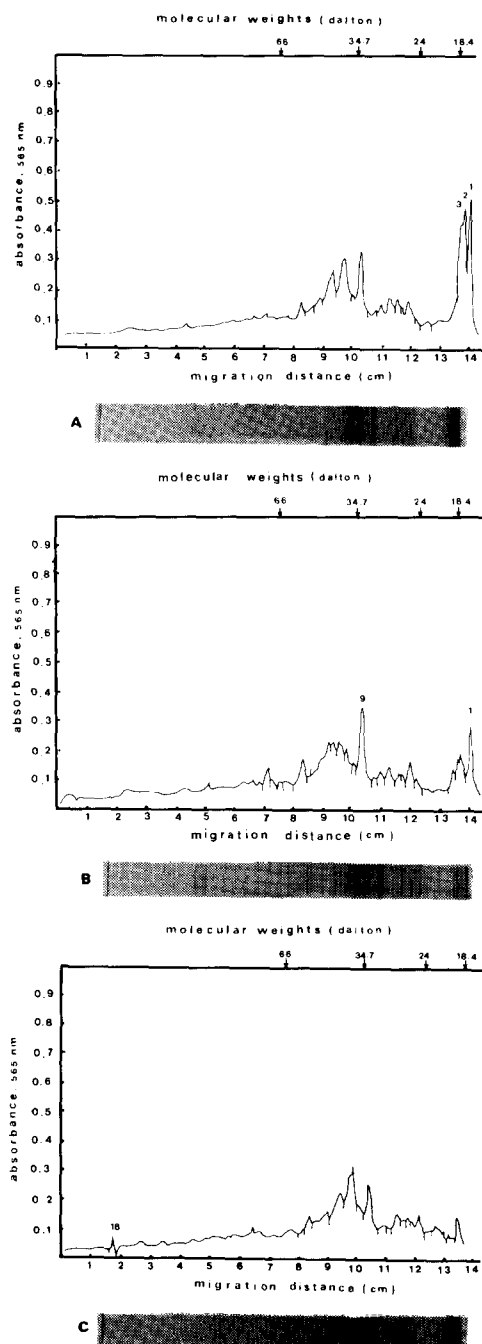


Fig. 13. Protein pattern of the cerebellum in 5-day-old. A. crude extraction, B. cytosolic fraction, C. membrane fraction.

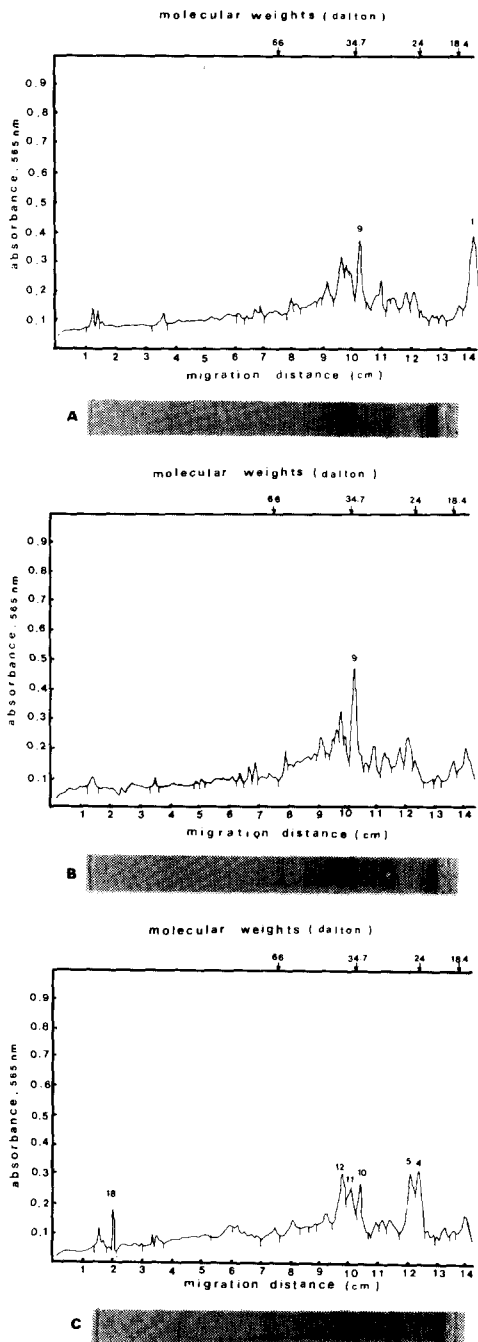


Fig. 14. Protein pattern of the cerebellum in 35-day-old. A. crude extraction, B. cytosolic fraction, C. membrane fraction.

essential for development, that the protein of band 18 was existent only the structural protein of membrane.

In the crude of 90-old-day cerebellum (Fig. 15), the protein of amount of bands was decreased than the 35-old-day protein, but only the protein of band 3 was increased. And the protein of amount of bands 4, 5, and bands 13-18 (49,200-260,600) were increased during development. In the cytosol of adult (Fig 15 B), the protein of bands 8 and 9 were shown abundant. While in the membrane, the protein of bands 13 and 14 (49,200, 63,800) appeared in large quantities (Fig. 15 C). Foregoing, the increasement of protein amount of bands through 49,200 dalton and 260600 dalton during development suggested that these protein were important regulatory protein.

By immunoblot analysis, the protein of crude are shown in lane 1 (Fig. 16). The cytosol appeared lane 2, and the lane 3 of membrane fraction was not almost detected (Fig. 16).

DISCUSSION

Raf oncogene family is *v-raf* which was isolated from a Murine Sarcoma Virus, 3611MSV, *a-*, *b-*, and *c-raf* activated genes that were related to the human disease²⁸⁾. And *d-raf* which was observed in *Drosophila Melanogaster* belong to *raf* family²⁸⁾. It was known that *a-* and *c-raf* encode cytoplasmic protein which are 68kd and 74 kd, respectively^{3,4,11,29)}. Also, *c-raf* of these was distributed almost in every tissues, especially, *a-raf* appeared higher level in epididymis²⁸⁾. *A-* and *c-raf* which was associated with a serine/threonine kinase have a function in signal transduction pathway through growth factors dependent downstream of the *ras* gene^{9,30)}, similar to physiological feature of *c-raf*. In the brain of *Geoclemys reevesii* which is a kind of the Reptiles, it was shown that *a-raf* labeled stronger than *c-raf*³¹⁾. It was just opposite to Mammalians.

In the cerebellum of rat, cells that labeled with the

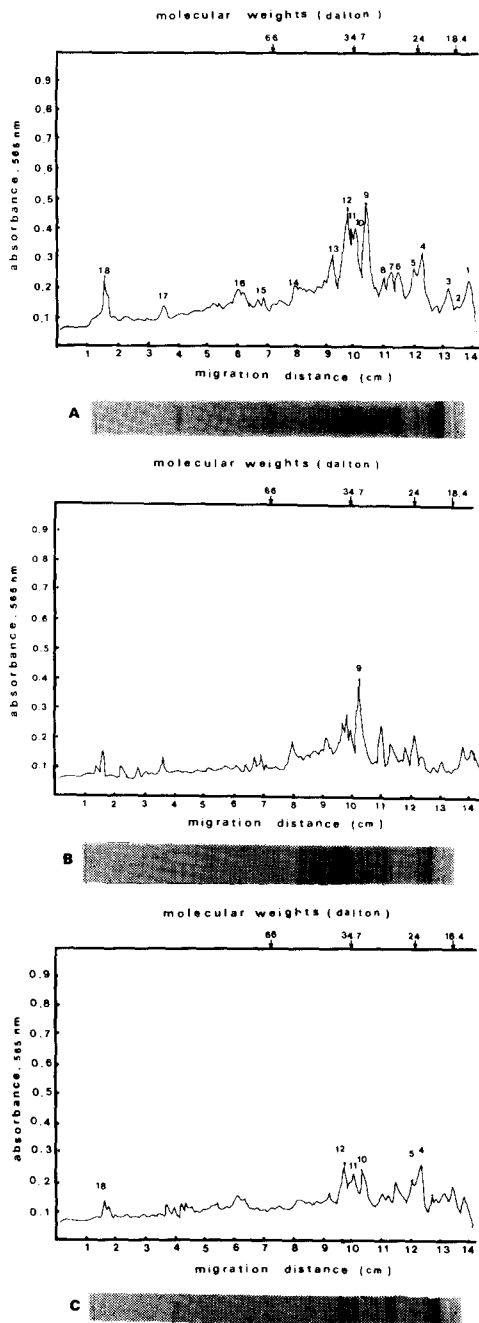


Fig. 15. Protein pattern of the cerebellum in 90-day-old.
A. crude extraction, B. cytosolic fraction, C. membrane fraction.

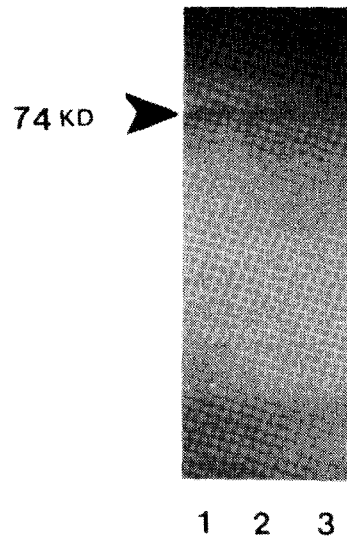


Fig. 16. Immunoblot of *raf* protein kinase in cerebellum.

1. crude extraction, 2. cytosolic fraction, 3. membrane fraction.

antibodies of *a-* and *c-raf* protein kinases detected easily at the cortex of cerebellum. But it was not detected in region which the neuronal fiber bundle pass. Therefore, in the brain of *Georchemyx revesii*, the region which was distributed cells labeled, was limited markedly, while in the cerebellum of rat, *a-* and *c-raf* protein kinase was distributed concentrically in *Pyramis cerebelli*, *Uvula*, *noulus*, *Paraflocculus*, *flocculus*, and *Nucleus emboliformis*. And it was shown that the cells in the purkinje cell layer and the cells of granular layer were labeled with antibodies.

As a mentioned, in the rat, *c-raf* protein kinase was labeled strongly than *a-raf*, accordingly, it supposed that *c-raf* protein kinase have a important of neurophysiology. Particularly, in the rat, the *raf* protein kinase was labeled concentrically in the purkinje cell. But cells of the granular layer was labeled weakly with *raf* protein kinase, and it was not almost observed that cells in molecular layer is labeled.

By the way, in NIH3T3 cell line which is fibroblast, *c-raf* protein kinase was translocated by treatment of platelet derived growth factor and phorbol ester³²⁾, this phenomena is a similar to the cases of PKC^{33,34,35,36)}. Also, PKC is a serine/threonine-specific protein kinase as like *raf* protein kinase, specially *a-* and *c-raf* protein kinase involved hyperconservative cysteine-rich region in amino-terminal which is observed on PKC^{12,13,14,15)}. By Girad et al.^{20,21)} Purkinje cells were densely labeled with PKC electrophoretically, it has been assumed that Purkinje cell was a function of presynapse of signal transduction in the cortex of the cerebellum. And PKC was distributed in perinuclear region of Purkinje cell, it has been suggested that PKC contribute to a variety of receptor-mediated biological signal transduction.

In this study, *raf* protein kinase is densely labeled in the cell body of Purkinje cells which exist in the cortex of cerebellum, this phenomena is coincided with the distribution of PKC in the Purkinje cell.

PKC was activated by diacylglycerol that produced in turnover of inositolphosphate when extra-signal was arrived on membrane^{37,38)}, then activated PKC enhanced the expression of *myc* gene which exist in nucleus³⁰⁾. Therefore, it had been supposed that *raf* protein kinase have a function of messenger in signal transduction by neurotransmitter as like PKC. However, it was represented that *raf* and *myc* act synergistically as inducing the carcinoma³⁹⁾. For that reason, it is known possibility that *raf* was interacted with PKC.

When signal was accepted by receptor in the membrane the activated *ras* that growth factor dependent tyrosine kinase was phosphorylated, was affected the translocation of *raf* into cytoplasm, by cascade, and *raf* affected the substance in nucleus. It was suggested by Rapp et al.³²⁾ that signal transduction may take place as above. Also, it was presumed whether *raf* is interacted with PKC, or a similar function with PKC, as related to happen carcinogenesis by which *raf* reacted synergistically with *myc*.

During the postnatal development of rat, it was shown that the growth of brain is the fastest between 2-old-day and 35-old-day^{40,41)}. Also, by Caley et al.⁴²⁾, it was represented that the cytoplasm and nucleus of the neuronal cell is morphologically changed during the growth of the brain of rat. But, as a result of this study, it was shown that the protein patterns of rat was gradually increased, especially, after 5-old-day the protein of higher molecular weight was increased in cytosol, and it was represented that the protein patterns of 35-old-day is a similar with 90-old-day. That is agreed with the fact was reported before.

And it was supposed that the increased protein is necessary to produce the neuronal cell in the neuronal system. In particular, the fact that the protein of lower molecular weight increased in cytosol, reflect that the protein is necessary to relate with the growth. But the protein of 260,600 dalton isolated in the membrane fraction is only existence in membrane, and I think that this protein was produced at embryo, located in the membrane of neuronal cell, and contributed to be necessary for signal transduction in the maturation and differentiation during the growth by receptor which associated with neuronal signal or membranous protein which was related to this receptor.

The protein was supposed to like that integral protein of 26,600 dalton in the membrane exist only in the Purkinje cell of cerebellum of mouse, and is regulated by cyclic AMP was found by Walass et al.⁴³⁾. Also, the increased protein of higher molecular weight between 49,200 dalton and 160,100 dalton is supposed signal transductant to related with lots of neurotransmitter.

And, in protein analysis using the immunoblot, *c-raf* protein kinase agree with the protein of 74,000 dalton by Rapp et al.^{4,30)}, *raf* represented a great deal in cytosol is known that found by immunocytochemical analysis is related with the distribution in neuronal cell particularly, Purkinje cells. Therefore, as above, *c-raf* protein kinase was distributed in a great deal of the cytoplasm

of the neuronal cell and Purkinje cell of cerebellum, this is assumed that is related to the signal transduction of neurotransmitter or other normal neuronal function.

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초록 : 쥐 소뇌에 있어서 *raf* protein kinases의 면역 세포 화학적 분포와 단백질 양상

박정순, 최원철†

(부산대학교 자연과학대학 생물학과)

본 연구에서는 SDS/polyacrylamide 젤 전기영동에 의한 쥐의 성장과정에 따른 소뇌의 단백질양상의 변화양상과 immunocytochemistry를 이용하여 *c-raf*, *a-raf* kinase의 정상 소뇌에서의 분포에 대해 관찰하였으며 western blot을 이용하여 소뇌의 단백질들에서 *c-raf*의 존재에 대해 살펴보았다.

단백질 양상에서 쥐의 성장에 따라 crude에서는 49,200 dalton과 169,000 dalton사이의 bands가 양적 증가를 보였으며 cytosolic fraction에서는 37,800 dalton의 band가 양적 증가를 보이는데 비해 membrane fraction에서는 260,600 dalton의 band가 증가하였다. 이러한 결과로 성장 발달에 따라 고분자량의 물질들이 이들 소뇌 부위에서 기여하였을 것으로 추정할 수 있었다.

Immunocytochemistry에 의한 분석에서는 *c-raf*와 *a-raf*가 소뇌의 피질부위에서 조롱박 세포(Purkinje cell)의 세포질 특히 핵 주변부위에서 강하게 검출되었으며 *a-raf*에 비해 *c-raf*가 더 강하게 나타났었다. 그리고 그 외에 *Nucleus emboliformis*의 큰 neuronal cell의 세포질 부위의 나타남을 볼 수 있었다.

Immunoblot에 의한 분석에서는 crude와 cytosolic fraction에서 *raf* protein kinase의 존재를 확인할 수 있었으며, 이상의 결과들을 종합해 보았을 때 소뇌의 정상의 많은 신경세포(neuronal cell)에 *raf* protein kinase가 분포되어 있으며 이들이 정상의 cell에서 기능을 가질 것으로 추정된다.