# Regulation of Pyruvate Kinase Type M2 by A-Raf: A Possible Glycolytic Stop or Go Mechanism

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**Abstract.** *Recently a link between A-Raf, cellular energy homeostasis and synthetic pathways has been suggested through the identification of pyruvate kinase type M2 (M2-PK), a key glycolytic enzyme, as interaction partner of A-Raf. In this study, we demonstrated that A-Raf is an important regulator of M2-PK function. In primary mouse fibroblasts, which are characterized by glutamine production and serine degradation, A-Raf induced dimerization and inactivation of M2-PK, thereby reducing conversion rates from glucose to lactate. In immortalized NIH3T3 fibroblasts, showing glutamine degradation and serine production, oncogenic A-Raf increased the highly active tetrameric form of M2-PK and favored glycolytic energy production. High serine levels thus may be responsible for the activation of M2-PK in A-Raf transformed NIH3T3 cells.*

Tumor growth is only possible if sufficient high energy phosphometabolites are available to meet the increased demand for energy and cellular building blocks, a regulatory mechanism termed the *metabolic budget system* (1). In proliferating cells, the intracellular phosphometabolites are regulated to a great extent by the glycolytic enzyme pyruvate kinase (EC 2.7.1.40). Pyruvate kinase (PK) catalyses the dephosphorylation of phosphoenolpyruvate (PEP) to pyruvate with the concomitant formation of ATP. Depending upon the metabolic functions of the tissues, different isoenzymes of pyruvate kinase are expressed. All proliferating cells express the pyruvate kinase isoenzyme type M2 (M2-PK), which can switch between a highly active tetrameric form, characterized by a high affinity to its substrate PEP, and a less active dimeric form with a low PEP affinity (1-5). The tetramer:dimer ratio is regulated by metabolic intermediates, such as fructose 1,6-P2 and serine, which induce an activation and tetramerization of M2-PK, but also by oncoproteins, such as the $pp60^{v\text{-}src}$ kinase and the HPV-16 E7 protein, which cause a dimerization of M2-PK (1, 6-9). The tetrameric form of M2-PK is a component of the glycolytic enzyme complex, which in addition to glycolytic enzymes (hexokinase, glyceraldehyde 3-P dehydrogenase, phosphoglycerate kinase, phosphoglyceromutase, enolase, pyruvate kinase, and lactate dehydrogenase) also contains components of the protein kinase cascade such as C-Raf, MEK and ERK, and AU-rich mRNA (8-13). In tumor cells, the dimeric form of pyruvate kinase accumulates and is referred to as *Tumor M2-PK*. The ratio between the tetrameric and dimeric form of M2-PK determines whether glucose carbons are degraded to pyruvate and lactate with the production of energy (tetrameric form) or channeled to synthetic processes, such as nucleic acid, amino acid and phospholipid synthesis (dimeric form) (1, 9, 14).

Preponderance of the dimeric form of M2-PK results in the requirement for additional energy sources. In tumor cells, the overexpression of glutaminase and NAD(P)-dependent malate decarboxylase gives rise to the possibility of a new energy producing pathway, the degradation of the amino acid glutamine to glutamate, aspartate and lactate,

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termed *glutaminolysis* (15-19). Another source for lactate production is the degradation of the amino acid serine to pyruvate and lactate termed *serinolysis* (8, 14).

A link between Raf kinase signaling and cellular energy metabolism has been provided through our previous identification of M2-PK as interaction partner for A-, but not C- or B-Raf in a yeast two-hybrid screen (20). Biochemical analysis of NIH3T3 cells expressing a constitutive active oncogenic form of A-Raf, gag-A-Raf (21), showed increased tetramerization of M2-PK and an increased glycolytic flux rate. In the work presented here we extend the analysis of metabolic control by A-Raf to wild-type as well as A-Raf deficient primary fibroblasts (22, 23).

## Materials and Methods

*Cell lines.* NIH3T3 6A cells were derived following transfection of parental NIH3T3 cells with a constitutively active transforming mutant of A-Raf (21). Primary fibroblast were obtained from wild-type and A-Raf deficient mice following standard procedures (22, 23). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum, and 100 U/ml penicillin/streptomycin in a humidified tissue culture incubator in a 5% $CO_2$/95% air mixture.

*Gel permeation and isoelectric focusing.* For gel permeation cells were extracted in lysis buffer containing 100 mM NaPi (pH 7.4), 1 mM DTT, 1 mM NaF, 1 mM mercaptoethanol, 1 mM ε-aminocaproic acid, 0.2 mM phenylmethylsulfonyl fluoride, 10% (v/v) glycerin. Extracts were passed over a gel filtration column and enzyme activities were measured in the fractions as described elsewhere (8).

For isoelectric focusing, cell extracts were prepared using a lysis buffer containing 10 mM Tris (pH 7.4), 1 mM NaF, 1 mM EDTA-$Na_2$ and 1 mM mercaptoethanol. Homogenates were subjected to free flow isoelectric focusing and enzyme activities were measured in the fractions as described elsewhere (8).

*Flux measurements.* For determination of nutrient conversion rates, glucose, glutamine, glutamate, lactate, alanine and serine were determined in the cell culture supernatants as described elsewhere (8).

*Coimmunoprecipitation.* HT 29 cells were homogenised in a lysis buffer containing 10 mM Tris/HCl (pH 7.5), 25 mM NaCl, 1 mM NaF, 0.2 mM phenymethylsulfonyl fluoride, 100 μM Na-vanadate, 10 μM glycerol phosphate, 10 μg/ml aprotinin and 0.1% NP40. Cell extracts were passed over DEAE-Sephacel from Sigma-Aldrich, Taufkirchen, Germany. The three fractions with the highest PK activity were pooled and subjected to immunoprecipitation using protein A/G plus agarose from Santa Cruz Biotechnology Inc, Heidelberg, Germany and monoclonal anti A-Raf antibodies from Sigma-Aldrich, Taufkrichen, Germany.

*Statistical analysis.* Statistical analyses were performed by one-factor (co)variance analysis with cell density as covariable using the software package BMDP. The artihmetic mean $\bar{x}\pm$SEM was applied to data, which showed a normal distribution of the measured variables. For ditributions skewed to the right, a logarithmic transformation of the data was performed and the results are presented as geometric mean and dispersion factor ($\bar{x}_g \bullet DF^{\pm 1}$). This is the de-logarithmic form of the arithmetic mean (± SD) of the previously logarithmically transformed data.

## Results

*Interaction between A-Raf and M2-PK.* To investigate the interaction between A-Raf and M2-PK co-immunoprecipitation experiments were performed from isoelectric focusing fractions of gag-A-Raf transformed NIH 3T3 cells. Association of A-Raf with M2-PK was detected in the fractions of the tetrameric but not the dimeric form of the enzyme (Figure 1A). In addition, immunoprecipitation of A-Raf from HT 29 cell lysates, which are known to contain high amounts of A-Raf also precipitated M2-PK, further demonstrating a direct interaction of A-Raf with M2-PK (Figure 1B and C). In contrast, in co-immunoprecipitation experiments performed under various conditions with lysates from K562 erythroleukemic cells or a K562 cell line overexpressing $(myc)_6$-A-Raf a direct interaction between A-Raf and M2-PK could not be demonstrated (data not shown).

*Effect of A-Raf on the activity and tetramer:dimer ratio of M2-PK.* Prior to the metabolic characterization, the levels of endogenous as well as exogenous A-Raf protein were analyzed in wild-type and A-Raf deficient mouse embryo fibroblasts as well as in immortalized NIH3T3 cells and NIH3T3 cells expressing an oncogenic A-Raf mutant. As shown in Figure 2, roughly equal amounts of the 68 kDa A-Raf protein were present in A-Raf wild-type primary fibroblasts, immortalized NIH 3T3 cells and gag-A-Raf transformed NIH 3T3 cells, whereas the transforming 85 kDa gag A-Raf protein was only expressed in gag-A-Raf transformed cells.

The analysis of A-Raf effects on the tetramer:dimer ratio of M2-PK yielded different results in primary fibroblasts compared to NIH3T3 cells. In wild-type primary fibroblasts, total PK activity was lower than in the A-Raf knockout counterparts (Figure 3). Both free flow isoelectric focusing and gel permeation experiments revealed a higher amount of the dimeric form of M2-PK in wild-type primary fibroblasts (isoelectric focusing: 31% dimer; gel permeation: 39% dimer) compared to A-Raf knockout primary fibroblasts (isoelectric focusing: 15% dimer; gel permeation: 18% dimer). In A-Raf knockout cells, the tetrameric form of M2-PK co-focused with glyceraldehyde 3-P dehydrogenase, phosphoglycerate kinase, enolase and nucleotide diphosphate kinase within the glycolytic enzyme complex. In fibroblasts wild-type for A-Raf, M2-PK migrated out of the glycolytic enzyme complex and focused at pI 7.2 (Figure 3). In NIH3T3 cells, which have intact wild-type A-Raf, transformation with oncogenic gag-A-Raf induced an increase of total pyruvate kinase activity as well as a tetramerization and migration of M2-PK into the glycolytic

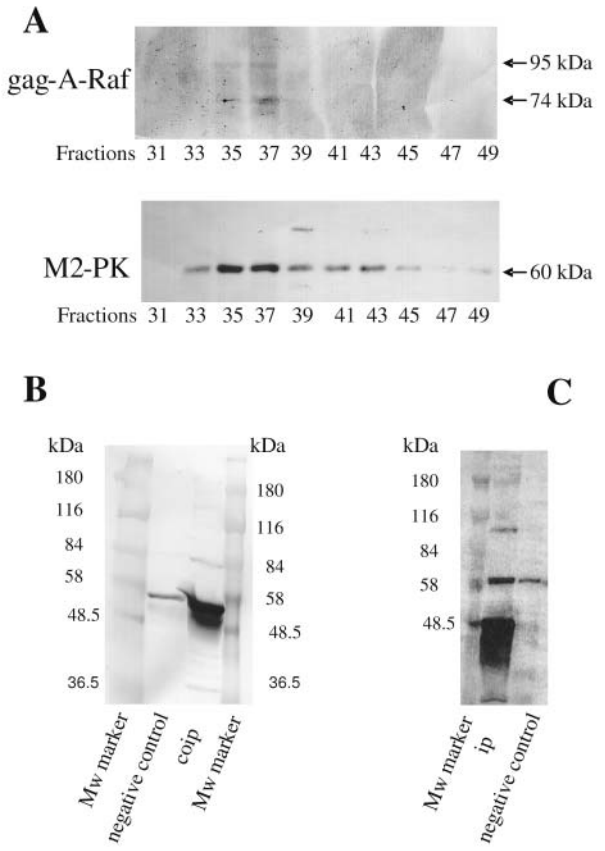


Figure 1. *Interaction between A-Raf and M2-PK. A) Fractions 31-49 of the isoelectric focusing experiment with gag-A-Raf transformed NIH3T3 cells (compare Figure 4) were subjected to immunoprecipitation with anti M2-PK antibody (DF4) from ScheBo Biotech, Giessen. M2-PK and co-precipitated gag-A-Raf was visualized after SDS-PAGE and electroblotting onto a nitrocellulose membrane using a biotinylated monoclonal anti M2-PK antibody (DF4) from ScheBo Biotech, Giessen or a polyclonal antiserum directed against p30gag, respectively. gag-A-Raf appears in two different forms: a glycosylated form of 90 kDa and a myristylated form of 75 kDa (21). B) HT 29 cell extracts were pre-purified with DEAE-Sephacel. A-Raf was immunoprecipitated using a monoclonal anti A-Raf antibody from Sigma. Co-precipitated M2-PK (coip) was stained in Western blots using a biotinylated monoclonal anti-M2-PK antibody. C) Control staining of immunoprecipitated A-Raf (ip) from experiment 1B in Western blots with monoclonal anti-A-Raf antibodies. In negative controls, immunoprecipitations were performed without antibody.*

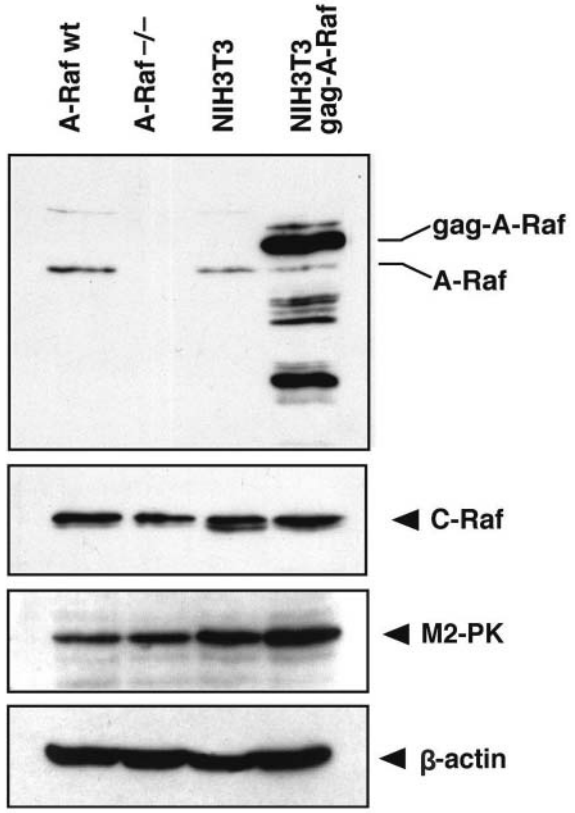


Figure 2. *Western blot analysis of the different fibroblast cell lines. Primary fibroblasts from wild-type and A-Raf knockout mice, as well as NIH3T3 cells and gag-A-Raf transformed NIH3T3 cells, were analyzed for the expression of A-Raf, c-Raf and M2-PK by Western blotting. β-actin was used as loading control. 100 μg protein/lane.*

enzyme complex in close proximity to glyceraldehyde 3-P dehydrogenase and enolase (Figure 4).

*A-Raf induced changes in M2-PK enzyme kinetics.* The differing PEP affinities of the tetrameric and dimeric form of M2-PK can be demonstrated by measuring PK activities at different PEP concentrations. Both, the tetrameric and dimeric form of M2-PK isolated from A-Raf knockout and A-Raf wild-type primary fibroblasts were highly active when measured in the presence of 2.0 mM PEP, which corresponds to the $v_{max}$ activity of pyruvate kinase (Figure 5A-C). A reduction of the PEP concentration to physiological levels (0.2 mM), led to an inactivation of the dimeric form, but only marginally affected the tetrameric form (Figure 5A and B).

High fructose 1,6-P2 levels induce a conversion of the dimeric to the tetrameric form of M2-PK (7, 24). *In vitro*, this effect can be simulated by the addition of fructose 1,6-P2 into the PK test assay. Interestingly, in A-Raf knockout fibroblasts, the dimeric form of M2-PK measured with 0.2 mM PEP could be reactivated by the addition of 1 mM fructose 1,6-P2 into the pyruvate kinase test assay, which was not possible in the A-Raf wild-type fibroblasts (Figure 5A and B). In both A-Raf knockout and A-Raf wild-type fibroblasts, alanine-induced inhibition of PK activity of the dimeric form could be overcome by serine (Figure 5C for A-Raf wild-type, and data not shown for A-Raf knockout cells).

*Effect of A-Raf on glycolytic, glutaminolytic and serinolytic conversion rates.* To further address metabolic processes in the different cells metabolic conversion rates were

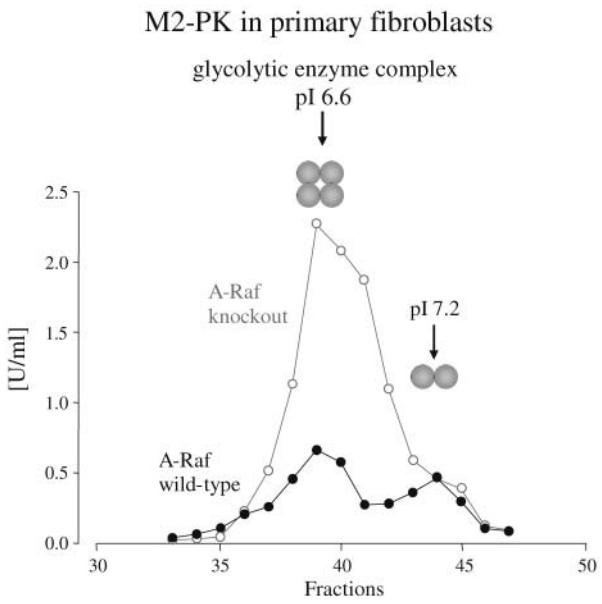


Figure 3. *Dimerization of M2-PK by wild-type A-Raf in primary fibroblasts. The tetrameric and dimeric forms of M2-PK were separated by free flow isoelectric focusing of primary fibroblasts from A-Raf knockout and A-Raf wild-type mice. In both cases same amounts of protein (8.5 mg (Biuret method)) were applied onto the column. Percentage of the dimeric form present: A-Raf knockout cells: 15%; A-Raf wild-type cells: 31%. In agreement with the isoelectric focusing data, gel permeation experiments also revealed a dissociation of the tetrameric to the dimeric form in A-Raf wild-type cells. Percentage of the dimeric form in gel permeation experiments: A-Raf knockout cells: 18%; A-Raf wild-type cells: 39%.*

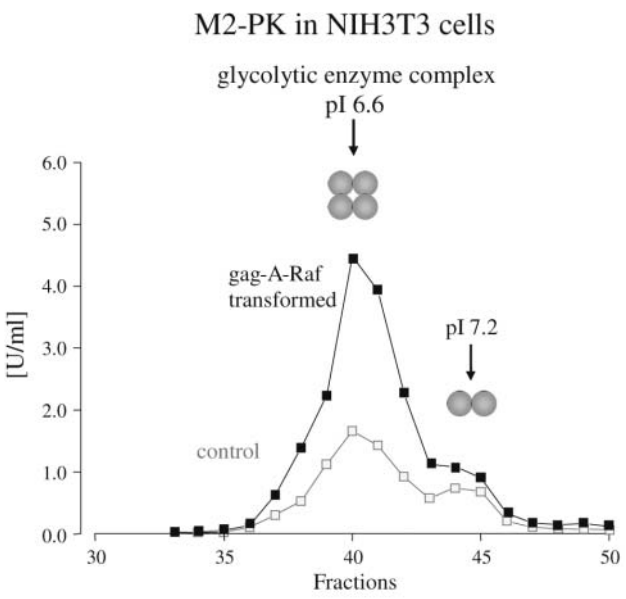


Figure 4. *Tetramerization of M2-PK in gag-A-Raf transformed NIH3T3 cells. The tetrameric and dimeric forms of M2-PK were separated by free flow isoelectric focusing of NIH3T3 cells and gag-A-Raf transformed NIH3T3 cell extracts. In both cases, extracts from 28 millions cells were applied onto the column. Percentage of the tetrameric form: in NIH3T3 cells: 69%; in gag-A-Raf transformed NIH3T3 cells: 76%. In agreement with the isoelectric focusing data, gel permeation experiments also revealed a tetramerization of M2-PK in gag-A-Raf transformed cells. Percentage of the tetrameric form in gel permeation experiments: in NIH3T3 cells: 77%; in gag-A-Raf transformed NIH3T3 cells: 87%.*

reconstructed from measurements of selected metabolites in the culture supernatants of the cells. Comparison of primary fibroblasts with NIH3T3 cells (Table IA and B) showed that parental and gag-A-Raf transformed NIH3T3 cells consumed glutamine, whereas in primary fibroblasts glutamine production was observed, indicating that in primary fibroblasts the glutaminolytic capacity was low.

In primary fibroblasts as well as NIH3T3 cells, rates of glucose consumption and lactate production correlated with the tetramer:dimer ratio of M2-PK. Due to the dimerization and inactivation of M2-PK in A-Raf wild-type primary fibroblasts, glucose consumption as well as lactate production decreased compared to the A-Raf knockout primary fibroblasts, which were characterized by a higher amount of the tetrameric form of M2-PK (Table IA). In the gag-A-Raf transformed NIH3T3 cells tetramerization and activation of M2-PK was accompanied by an increase of glucose consumption as well as lactate production compared to parental NIH3T3 cells in which a higher amount of the dimeric form was found (Table IB).

Another source for lactate production is serinolysis, whereby the amino group of serine is transferred to pyruvate with the production of alanine (Figure 6) (8, 14). The remaining carbons are converted to glycerate 2-P and degraded to pyruvate and lactate within the lower part of the glycolytic pathway (enolase and pyruvate kinase reactions). Determination of serine flux rates revealed an interesting correlation between the cell lines and the conversion rates of serine (Figure 7). In A-Raf knockout primary fibroblasts, an increase in serine consumption correlated with an increase in lactate and alanine production, which corresponds to a degradation of serine to lactate (Figure 7A and B). In A-Raf wild-type fibroblasts, the serinolytic flux rate was reduced as reflected by a flatter slope between serine consumption and lactate as well as serine consumption and alanine production. In control NIH3T3 cells, no correlation was found between the conversion rates of serine and the production rates of lactate and alanine. In gag-A-Raf transformed NIH3T3 cells, increased rates of lactate and alanine production correlated with an enhanced rate of serine production, pointing to a turnaround in serine metabolism from consumption to serine production.

Serine production starts with the glycolytic intermediate glycerate 3-P (Figure 6). In accordance, the stepwise turnaround of serine metabolism from serine degradation

to serine production was also reflected by a turnaround in the correlation between glucose consumption and serine conversion. In primary A-Raf knockout fibroblasts, in which serine consumption correlated with lactate production, an increased rate of glucose consumption was accompanied by an increased rate of serine consumption (slope=0.2; r=0.740). In the gag-A-Raf transformed NIH3T3 cells, where serine conversion shifted from consumption to production an increase in glucose consumption correlated with an increase in serine production (slope=0.08; r=0.519). The correlation between glucose consumption and serine production points to an increased channeling of glucose carbons to serine synthesis in gag-A-Raf transformed NIH3T3 cells.

## Discussion

In comparison to A-Raf knockout primary fibroblasts, A-Raf wild type primary fibroblasts were characterized by a shift of M2-PK to the dimeric form and a migration out of the glycolytic enzyme complex (Figure 3). On the other hand, in NIH3T3 cells, which were charaterized by a high amount of the tetrameric form of M2-PK, transformation with gag-A-Raf correlated with a further increase of the tetrameric form and a migration of M2-PK into the glycolytic enzyme complex (Figure 4). The close spatial proximity of the enzymes within the glycolytic enzyme complex allows a very effective conversion of glucose to pyruvate and lactate (8, 9, 12). Accordingly, in wild-type A-Raf fibroblasts, dimerization and inactivation of M2-PK was accompanied by a decrease of glucose consumption and lactate production whereas tetramerization and activation of M2-PK in gag-A-Raf transformed NIH3T3 cells correlated with an increase in glucose consumption and lactate production (Table IA and B).

The most interesting finding was the stepwise turnaround in serine metabolism from serine degradation (serinolysis) to serine production in A-Raf knockout fibroblasts, A-Raf wild-type fibroblasts, immortalized parental NIH3T3 and gag-A-Raf transformed NIH3T3 cells (Figure 7). Serine itself and alanine, which is the acceptor of the serine amino group in serine degradation, both directly influence the activity of M2-PK. Whereas alanine leads to an inhibition of M2-PK, serine induces an activation of the enzyme (1, 9). Interestingly, kinetic characterization of M2-PK revealed that, contrary to A-Raf knockout cells in A-Raf wild-type cells, the dimeric form of M2-PK could no longer be activated by fructose 1,6-P2 but was very well activatable by serine (Figure 5). This result indicates that A-Raf may control the susceptibility of M2-PK to metabolic regulation from fructose 1,6-P2 to serine. Therefore, the tetramerization of M2-PK found in gag-A-Raf transformed NIH3T3 cells might have been the result of a secondary metabolic effect induced by the turn in

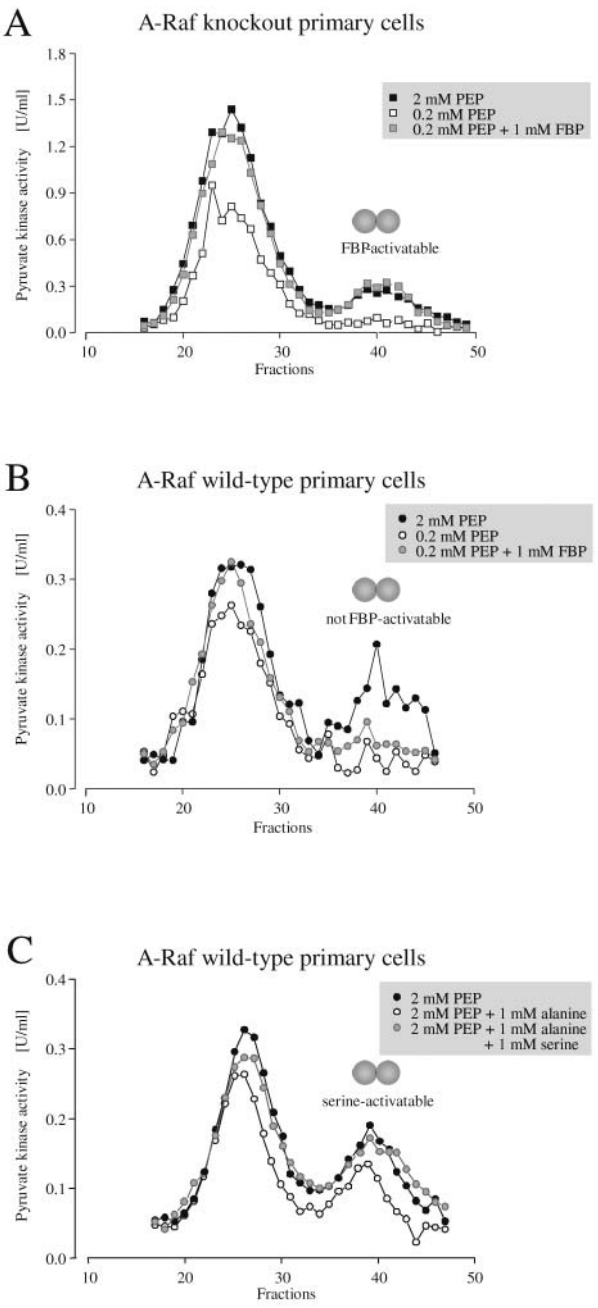


Figure 5. *Comparison of the capacities of fructose 1,6-P2 and serine to activate the dimeric form of M2-PK in A-Raf knockout and A-Raf wild-type primary fibroblasts. Cell extracts of A-Raf knockout and A-Raf wild-type primary fibroblasts were passed over a gel permeation column in order to separate the tetrameric and dimeric form of M2-PK. Pyruvate kinase activities in the fractions were measured in the presence of 2 mM PEP which correspond to $v_{max}$ activities of the enzyme and with 0.2 mM PEP which correspond to physiological conditions. A) and B) The potential of fructose 1,6-P2 to activate the dimeric form of M2-PK was measured in the presence of 0.2 mM PEP and 1 mM fructose 1,6-P2. C) The potential of serine to activate the dimeric form of M2-PK was measured with 2 mM PEP, 1 mM alanine and 1 mM serine. Alanine inhibits PK $v_{max}$ activity (2 mM PEP). In A-Raf wild-type and A-Raf knockout cells, alanine inhibition of the dimeric form of M2-PK was overcome by serine.*

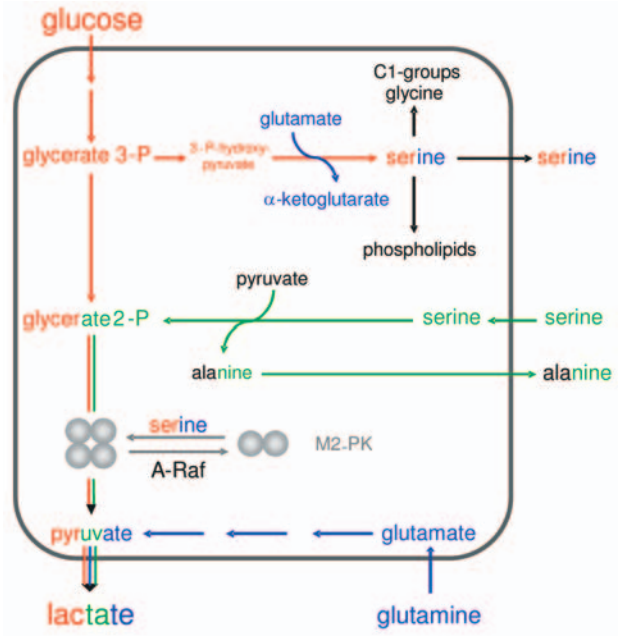


Figure 6. *Interaction between glucose, glutamine and serine metabolism. Red: glycolysis, blue: glutaminolysis, green: serinolysis.*

serine metabolism from catabolism (serinolysis) to anabolism (serine production). Serine is an essential precursor for glycine and activated methyl groups used in the synthesis of purines and pyrimidines as well as of phospho- and sphingolipids (Figure 6). However, if the supply of active methyl groups from the serine pool is too high, the tetrahydrofolate is irreversibly channeled to N5-methyl-tetrahydrofolate, the *methyl trap*, and not further available for nucleic acid synthesis (1). Therefore, the activation of M2-PK by serine is an effective regulatory feedback mechanism to prevent serine overproduction and the methyl trap. The turnaround in serine metabolism could depend on differences in glutamine metabolism. Primary fibroblasts produced glutamine, whereas immortal NIH3T3 cells were characterized by high glutamine consumption rates (Table IA and B). Glutamine and its degradation product glutamate are the donors of the amino group for the synthesis of serine (Figure 6). A high glutaminolytic capacity is necessary for cell transformation and tumorigenesis (15-19, 25).

Taken together the different metabolic effects of A-Raf in primary fibroblasts and NIH3T3 cells indicate that the effects of A-Raf on M2-PK depend on the basic metabolic characteristics of the individual cell line, which are summarized as the *metabolome* of the cells. Depending upon the metabolome of the parental cell line, expression of A-Raf was found to induce a glycolytic "stop" (dimerization and inhibition of M2-PK; decrease in glucose consumption and lactate production) as well as a glycolytic "go" (tetramerization and activation of M2-PK; increase in glucose consumption and lactate production).

Table I. *Glycolytic and glutaminolytic conversion rates (A) in A-Raf knockout and A-Raf wild-type primary fibroblasts as well as in (B) non-transformed and gag-A-Raf transformed NIH3T3 cells.*

Table IA.

| Metabolite | | A-Raf knockout primary cells | A-Raf wild-type primary cells | Significance |
|---|---|---|---|---|
| Consumption | Production | nmoles/h | $\bar{x}$ ±SEM | |
| Glucose | | 55.0±8.4 (10) | 31.2±4.4 (10) | <0.01 |
| | Glutamine | 8.9±6.8 (10) | 12.3±7.1 (10) | <0.01 |
| | Lactate | 81.9±2.2 (10) | 61.1±1.3 (10) | <0.001 |

Table IB.

| Metabolite | | NIH3T3 cells | gag-A-Raf transformed NIH3T3 cells | Significance |
|---|---|---|---|---|
| Consumption | Production | nmoles/h | $\bar{x}_g \bullet DF^{\pm 1}$ | |
| Glucose | | 24.0 • 1.2 (9) | 57.5 • 1.2 (9) | <0.01 |
| Glutamine | | 4.4 • 1.2 (10) | 11.5 • 1.2 (10) | <0.01 |
| | Lactate | 52.5 • 1.0 (10) | 79.4 • 1.0 (10) | <0.001 |

Glucose, glutamine and lactate conversion rates were measured in the culture supernatants of the cells. The data reflect conversion rates accomplished by 100.000 cells. ($\bar{x}_g \bullet DF^{\pm 1}$) corresponds to the de-logarithmic form of the arithmetic mean (±SD) as described in Materials and Methods. In parentheses: number of experiments.

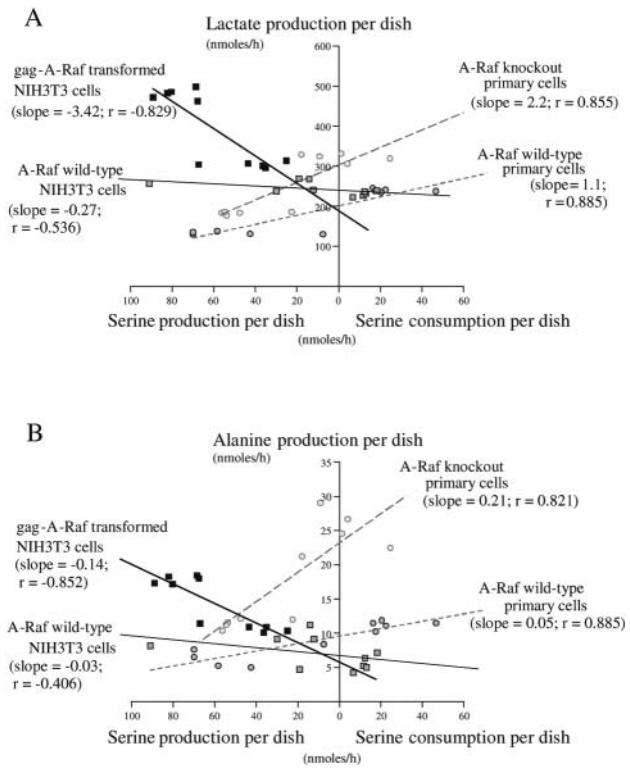


Figure 7. *Stepwise turnaround in serine metabolism from serinolysis to serine production in primary fibroblasts and NIH3T3 cells. A) Correlation between serine conversion and lactate production rates. B) Correlation between serine conversion and alanine production rates. Serine, lactate and alanine conversion rates were measured in the cultivation supernatants of the cells.* ○ *= A-Raf knockout primary cells;* ● *= A-Raf wild-type primary cells;* ■ *= A-Raf wild-type NIH 3T3 cells;* ■ *= gag-A-Raf transformed NIH3T3 cells.*

A dependency of metabolic effects on the basic metabolic characteristics of the cells has also been found for the interaction of the E7 oncoprotein of the human papilloma virus type 16 (HPV-16 E7) with M2-PK in different NIH3T3 cell strains (8). In high glycolytic NIH3T3 cells, which are characterized by a high amount of tetrameric form of M2-PK and a low glutaminolytic flux rate, E7 transformation led to a dimerization of M2-PK. In contrast, in the low glycolytic NIH3T3 cell strain which is characterized by a high amount of the dimeric form of M2-PK and a high glutaminolytic flux rate, E7 transformation did not influence the tetramer:dimer ratio of M2-PK.

Currently there is a lack of understanding as to exactly how A-Raf controls the activity and aggregation state of M2-PK. We have shown that A-Raf, but not B- or C-Raf, interacts with M2-PK in a yeast two-hybrid screen (20). Binding between A-Raf and M2-PK was also found by co-immunoprecipitations in gag-A-Raf transformed NIH3T3 and in HT29 cells (Figure 1). However, in K562 erythroleukemic cells, which were studied as a third model system, this interaction could not be demonstrated. Currently the molecular basis for this observation is unclear and remains to be investigated. Cell type-specific properties may at least partially account for this result. In our previous work, we found that the oncogenic potential of A-Raf was severely compromised when coexpressed together with a kinase dead mutant of M2-PK (20). Therefore, it remains to be seen whether physical interaction between the two proteins is a prerequisite for the A-Raf effects described here. Phosphorylation has been implicated in the regulation of M2-PK before, and in different tumor cell lines an increased phosphorylation of M2-PK in serine and tyrosine has been described (6, 27-29). For tyrosine phosphorylation of M2-PK, $pp60^{v\text{-}src}$ has been implicated. In $pp60^{v\text{-}src}$ kinase transformed cells, the increased tyrosine phosphorylation of M2-PK correlates with a dimerization and inactivation of the enzyme which results in an expansion of phosphometabolite pools, such as fructose 1,6-P2 and P-ribose PP, which are then available as precursors for synthetic processes (6). Serine phosphorylation of M2-PK in tumor cells was shown to be cAMP-independent and inducible by EGF or PKCδ (27, 29, 30). In NIH3T3 cells, we observed a 1.9 fold increase in serine phosphorylation of M2-PK in the presence of activated A-Raf (data not shown).

The results of our study establish a link between Raf kinases and cellular metabolism, which may be essential for the Raf control of cell proliferation and survival (31). While the interaction with M2-PK is specific for A-Raf, only B- and C-Raf so far have been found to be involved in human tumor development (26, 32, 33). However, transformation potential has been demonstrated before for artificially activated A-Raf (gag-A-Raf) (21, 34) and evidence for crosstalk between various Raf isoforms, which may lead to activation, has been demonstrated for B- and C-Raf, as well as for C- and A-Raf (35-37). We also have shown that a dominant negative mutant of M2-PK was able to block A-Raf transformation (20). Given the tremendous interest in Raf-directed therapies, the signaling connection suggested here may provide novel possibilities for the treatment of tumors characterized by hyperactive Raf signaling resulting from mutations in Raf or upstream components (26, 38-40).

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