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PLASMA MEMBRANE PROTEIN KINASE ACTIVITY IN NORMAL AND ROUS SARCOMA VIRUS-TRANSFORMED CHICK EMBRYO FIBROBLASTS

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Summary

A preliminary study has been carried out to investigate the effect of Rous sarcoma virus transformation on plasma membrane protein kinase activity in chick embryo fibroblasts. Enzyme activity was measured using an in vitro phosphorylation method employing [γ-^{32}P]ATP with isolated plasma membranes serving as the source of both protein kinase and protein substrate. In general, the enzymatic properties observed were similar to those of other known protein kinases. However, for maximal activity a marked dependence on high Mg^{2+} concentrations was noted. Evidence was obtained which showed that cyclic nucleotide-dependent protein kinases were present in membranes from normal cells, but none could be measured in preparations from transformed cells. In addition, transformation appeared to result in a slight increase in basal plasma membrane protein kinase activity.

Introduction

There is considerable evidence to support the view that the cell surface is involved in the regulation of cell growth and thus it has been proposed that alterations in the plasma membrane may underlie changes in the growth properties of virus transformed and cancer cells (cf. ref. 1). One particular system, that of chick embryo fibroblasts transformed by Rous sarcoma virus (RSV), has been widely studied primarily because of the high frequency of transformation (almost 100%) making cell cloning unncessary, and the availability of

Abbreviations: ATP: adenosine triphosphate; cyclic AMP: adenosine 3′,5′-monophosphate; cyclic GMP; guanosine 3′,5′-monophosphate; GTP: guanosine triphosphate; RSV: Rous sarcoma virus; SDS: sodium dodecyl sulfate; ts 68: temperature sensitive mutant 68 of RSV.

viral mutants which are temperature-sensitive for the maintenance of the transformed phenotype. A number of alterations in cell surface proteins have been reported [2—9]; however, direct evidence linking these changes with alterations in cell growth properties is in general lacking.

It is possible that more subtle modifications may play an important role in regulating plasma membrane functions. One such mechanism concerns the action of protein phosphorylation-dephosphorylation systems at the cell surface. The biological activity of many proteins can be altered by phosphorylation (e.g., see reviews [10—12]) and thus it is possible that changes in the phosphorylation of plasma membrane structural proteins, enzymes or mitogen receptors may significantly control cell proliferation.

Protein kinases which catalyse protein phosphorylation are present as cytosol, nuclear, and plasma membrane-bound enzymes. The activity of one class of protein kinases can be controled through an interaction with cyclic AMP which results in the separation of the regulatory and catalytic subunits of the enzyme producing an increase in enzyme activity (ref. 13—16, cf. refs. 10—12). Cyclic GMP-dependent protein kinases [17] and cyclic AMP-dependent protein phosphatases [18—19] have also been reported and thus it is possible that protein phosphorylation and dephosphorylation may be regulated at least in part via changes in cyclic nucleotides.

Changes in intracellular levels of cyclic nucleotides appear to play a role in the control of cell proliferation in both normal and virus transformed cell populations [20—23]. In particular, studies on chick embryo fibroblasts have generally indicated a significant reduction in cyclic AMP levels following transformation by RSV (e.g. refs. 24—25). Since the activation of protein kinases is clearly the best understood biochemical effect of cyclic nucleotides, it is possible that the mechanism by which cyclic nucleotides regulate cell function is via the protein phosphorylation-dephosphorylation system.

Thus far few studies have dealt with the role of protein phosphorylation in viral transformation. In the present study we report initial experiments on plasma membrane protein kinases of normal and RSV transformed cells using an in vitro assay system.

Materials and Methods

Cell culture and viruses

Eleven-day old COFAL negative chick embryos (Spafas, Norwich, Conn., U.S.A.) were used to prepare primary cultures. All experiments were performed on secondary cultures growing on 100-mm plastic dishes (Corning, N.Y., U.S.A.) using medium 199 containing 10% tryptose phosphate broth, 4% calf serum and 1% heat-activated chick serum (all from GIBCO, Grand Island, N.Y., U.S.A.), as previously described [3]. For transformation studies both wild-type Schmidt-Ruppin (subgroup A) and a mutant ts 68 (cf. ref. 26) derived from this same wild type strain, were used. This mutant has been shown to contain a thermosensitive lesion in the viral gene required for transformation such that infected cells were phenotypically transformed when incubated at 36°C, but were phenotypically normal at 41°C. Virus production has been shown to be equivalent at the two incubation temperatures [26]. Primary

cultures were infected with about 1 focus-forming unit of virus per cell and after 2—3 days at 36°C the cells were subcultured and experiments were carried out on secondary cultures incubated at the appropriate temperature. The medium on all cultures was changed daily.

Isolation of plasma membranes

Plasma membranes were isolated by the method of Brunette and Till [27] with the introduction of slight modifications as previously described [3,28]. The addition of Zn^{2+} (micromolar amounts) as a 'hardening' agent during membrane isolation was employed. No chelating agents were added to remove these ions. This method routinely provided material with a 6—10-fold increase in the specific activity of 5'-nucleotidase, and with a 3—5-fold increase for ouabain-sensitive ($Na^+ + K^+$)ATPase. There was a consistent low level contamination by endoplasmic reticulum as measured by DPNH-diaphorase activity (ref. 28 and Branton, P.E., unpublished). Soluble proteins were virtually absent. The polypeptide pattern of such membranes as determined by SDS polyacrylamide gel electrophoresis was virtually indistinguishable from that obtained by another method (i.e. ref. 29; see ref. 8 and 3), although a higher quantity of the external LETS protein (cf. ref. 2, 3 and 6) was retained using the present procedure. Membrane preparations were generally used immediately after isolation, however in some experiments frozen material was stored at −70°C until use (never more than 4 weeks). All comparisons between normal and transformed cells were made with membrane preparations isolated simultaneously from cultures prepared from the same set of embryos.

In vitro assay for plasma membrane protein kinase

Plasma membrane protein kinases were assayed in vitro by a method adapted from the group of Greengard [30]. A standard reaction mixture of 200 μl was used which contained 100 μg of plasma membrane protein, 10 μmol of sodium acetate (pH 6.0), 2 or 20 μmol of $MgCl_2$ (10 and 100 mM, respectively), theophylline (8 μM), NaF (40 μM), and 1.5 nmol of ATP. The ATP used was a mixture of [γ-^{32}P]ATP (New England Nuclear, Boston, Mass. U.S.A.; specific activity 10—35 Ci/mmol) diluted in non-radioactive ATP to a final specific activity of 2—10 Ci/mmol. In some experiments, cyclic AMP, cyclic GMP, or dibutyryl cyclic AMP were added directly to the reaction mixture to the final concentrations described in individual experiments. Reactions were carried out at 30 or 37°C for 2 min. The reaction was stopped by the addition of 2 ml of cold 10% trichloroacetic acid (w/v in 0.033 M H_3PO_4). A 0.2 ml-aliquot of 0.1% bovine serum albumin (w/v) was added as carrier, the samples were chilled in ice for 1—16 h and then they were filtered using glass fibre filters (Whatman, Clifton, N.J., U.S.A.) with a 20-ml wash of 5% trichloroacetic acid (w/v in 0.017 M H_3PO_4) and a 10-ml wash of 95% ethanol. The filters were dried and counted using Triton/toluene scintillation fluid (Formula 950^A-New England Nuclear) in a Beckman Liquid Scintillation Counter. The radioactivity detected was used to calculate the number of pmol of phosphate incorporated. Protein concentrations were determined by the method of Lowry et al. [31]. In experiments in which purified beef heart protein kinase was employed, the reaction conditions were the same as described above except that no membrane was present and

typically 5 μg of protein kinase was added along with 100 μg of arginine-rich histone to serve as substrate.

Theophylline, ATP, cyclic AMP, cyclic GMP, dibutyryl cyclic AMP, cyclic AMP-dependent beef heart protein kinase, arginine-rich histone, pronase, adenosine, cordycepin, deoxyribonuclease, and ribonuclease were all purchased from Sigma (St. Louis, Mo., U.S.A.). All other chemicals were purchased either from British Drug House (Poole, England) or Fisher Scientific (Fair Lawn, N.J., U.S.A.).

The experimental error found in all quantitative studies was consistently between 8—13%.

Results

Experimental conditions for protein kinase assay of plasma membranes from normal and RSV-transformed chick embryo fibroblasts

Since plasma membrane protein kinase from chick embryo fibroblasts had not been previously analyzed, a series of experiments was performed to optimize the reaction conditions for the in vitro assay. The data to be presented was obtained from experiments using membranes from normal and transformed cells assayed in the absence of cyclic nucleotides. However qualitatively similar results were obtained in the presence of exogenous cyclic AMP and cyclic GMP.

First, the dependence of in vitro protein phosphorylation on the concentration of membrane protein was determined over a range of 0—400 μg per

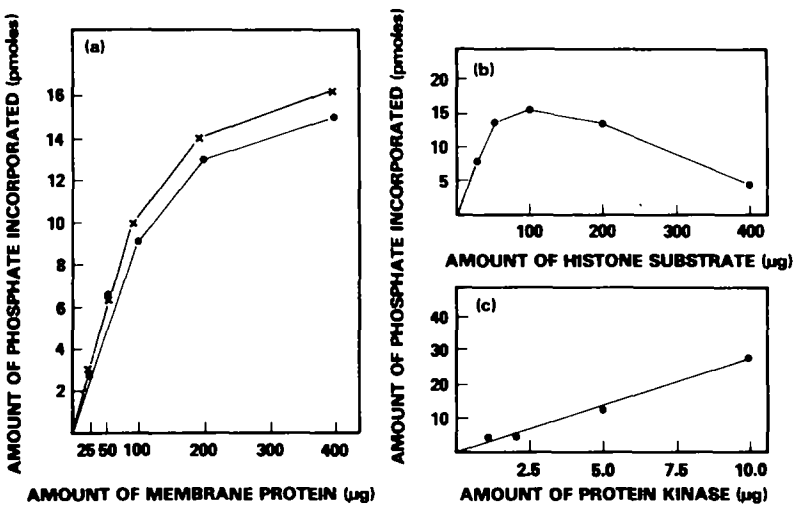

Fig. 1. Effect of protein concentration on protein kinase activity. The in vitro phosphorylation reaction was carried out on plasma membranes purified from normal (●———●) or RSV-transformed (X———X) cells under standard conditions outlined in Materials and Methods except that the quantity of membrane protein present was varied as indicated in a. In addition the standard assay was carried out using purified beef heart protein kinase with arginine-rich histone as substrate. In one series of experiments 5 μg of protein kinase was reacted with various amounts of histone substrate as indicated in b. In another series the amount of histone substrate was kept constant at 100 μg and the amount of protein kinase varied as shown in c.

reaction. The results shown in Fig. 1A indicated that enzyme activity in both normal and transformed cell preparations increased directly with protein concentration up to about 100 μg per reaction. At higher amounts of protein the rate of reaction decreased. This decrease may have been due to a number of reasons, the most likely being interference of the reaction due to overloading of the incubation mixture. In parallel experiments using purified beef heart cyclic AMP-dependent protein kinase, the reaction rate remained constant with increasing enzyme concentrations over a wide range, as shown in Fig. 1C. However, when this purified enzyme was incubated with increasing amounts of histone substrate, a decreased reaction rate was seen in excess of 100—200 μg histone, as shown in Fig. 1B. Thus is seemed likely that interference occurred when high protein concentrations were used. It is also possible that the presence of protein phosphatases or perhaps protein kinase inhibitors in the membrane preparations also contributed to the decline. For future analyses 100 μg of membrane protein per reaction was chosen as standard since this amount provided maximum activity in the linear range of the assay.

The effect of varying pH (5—7.5), temperature (25—56°C) and concentration of ATP (0—5 nmol per reaction) was also determined. The results obtained (data not shown) were identical for both normal and transformed cell plasma membranes. The rate of reaction was highest between pH 6—7 and a pH of 6.0 was selected for future experiments. The temperature optimum was between 30 and 39°C and thus 30 or 37°C was used for further studies. Activity increased with increasing ATP concentration and 1.5 nmol (7.5 μM) was chosen for all future experiments. In addition it was shown that incorporation of ^{32}P was due to protein kinases and not to phosphoprotein kinases which can utilize GTP as a phosphate donor. Experiments in which non-radioactive GTP was added in 10-fold excess to [γ-^{32}P]ATP indicated that no reduction of ^{32}P incorporation occurred (data not shown).

When working with unpurified enzymes the level of phosphorylation of proteins by protein kinases may depend upon two factors: the rate of phosphorylation, and the rate of dephosphorylation caused by phosphatases. One must therefore choose a reaction time which provides high levels of labeling and with low dephosphorylation. The standard in vitro labeling procedure was carried out except that the period of incubation was altered. The results shown in Fig. 2 indicated that the rate of incorporation was linear for about 5—10 min, after which time incorporation dramatically decreased. In parallel experiments (also shown in Fig. 2) using purified beef heart protein kinase, the rate of reaction was constant up to at least 30 min. The decrease seen with plasma membranes was undoubtedly due to the action of membrane-bound ATPase and to endogenous protein phosphatases which were probably not present in the purified protein kinase preparation. All future quantitative analyses were therefore carried out using a 2-min period of incubation to ensure a linear rate of incorporation.

It is well known that protein kinases require the presence of divalent cations and in a great number of previous studies soluble enzyme has been shown to exhibit maximum activity at low concentrations of Mg^{2+}, in the order of 10 mM. In an experiment with the commercial purified beef heart protein kinase the data obtained, shown in Fig. 3, also indicated a fairly sharp magnesium ion

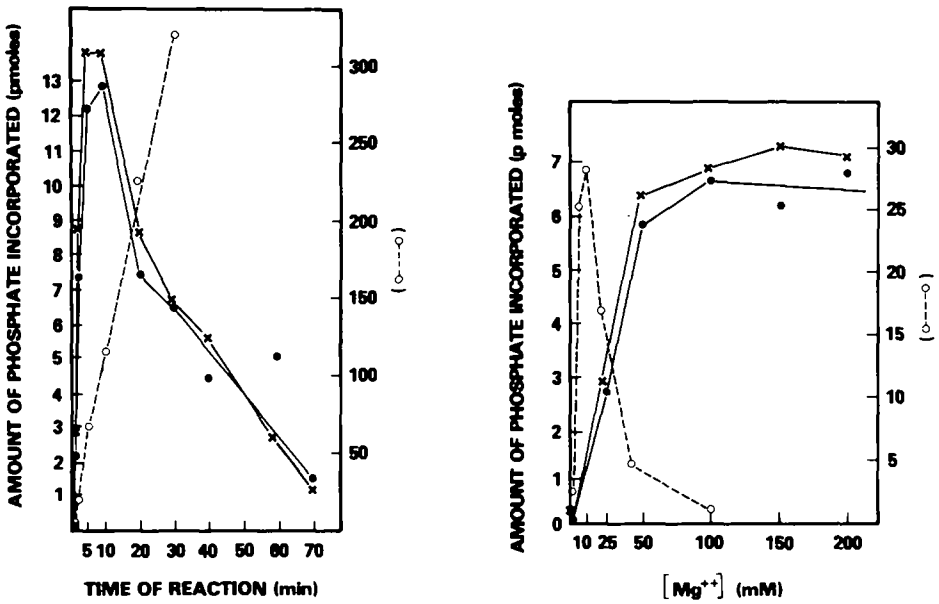

Fig. 2. Effect of period of incubation on protein kinase activity. The in vitro phosphorylation reaction was carried out under standard conditions except that the period of incubation was varied as shown in the figure. Plasma membranes from normal (●———●) or RSV-transformed (X———X) cells. Purified beef heart protein kinase with arginine-rich histone as substrate (○- - - - -○).

Fig. 3. Effect of Mg^{2+} concentration on protein kinase activity. The in vitro phosphorylation reaction was carried out under standard conditions except that the final concentration of $MgCl_2$ present was varied as shown in the figure. (●———●) normal and (X———X) RSV-transformed cell plasma membranes. (○- - - - -○), purified beef heart protein kinase with arginine-rich histone as substrate.

optimum at about 10 mM. Plasma membrane protein kinases were found to behave quite differently however. As also shown in Fig. 3, there was a marked dependence of the reaction on Mg^{2+}. The maximum reaction rate was attained at a concentration of 50—100 mM and the rate remained constant up to 200 mM. For most future experiments two Mg^{2+} concentrations were used, 10 and 100 mM.

Although Mg^{2+} has been used in the present studies, other divalent cations can support protein kinase activity. In another series of experiments (data not shown) using membranes from normal and RVS-transformed cells a large number of cations have been tested in the in vitro reaction. In addition to magnesium, Mn^{2+}, Co^{2+}, Fe^{2+}, and Ni^{2+} were found to support high enzyme activity; Zn^{2+} and Cu^{2+} allowed somewhat lower levels of incorporation; and with Ca^{2+}, K^+ and Na^+ essentially no protein kinase activity could be detected at all. Of particular relevance in the present study was the action of Zn^{2+}, since $ZnCl_2$ (in micromolar amounts) has been used in the preparation of the isolated membranes. It is unlikely that small amounts of Zn^{2+} retained in the membranes have greatly affected the reaction since in other experiments (not shown) it was found that enzyme activity measured in the presence of mmolar amounts of Mg^{2+} was unchanged by the addition of μ molar amounts of Zn^{2+}.

Finally, to ensure that the assay system was in fact measuring the phosphorylation of proteins by active protein kinases, a number of control studies

were also carried out (data not shown). Heat treatment (100°C for 2 min) of membranes prior to incubation reduced ^{32}P incorporation by 98%. The use of inorganic $^{32}PO_4$ or [α-^{32}P]ATP (New England Nuclear) instead of [γ-^{32}P]ATP resulted in virtually no incorporation of ^{32}P. Pronase treatment (100 μg/ml) and mild alkaline hydrolysis following incubation removed over 90% of all labeled material, but deoxyribonuclease and ribonuclease treatment (100 μg/ml) or chloroform/methanol extraction removed less than 2%. Finally, the addition of adenosine or cordycepin ($4 \cdot 10^{-4}$ M) to the reaction mixture reduced ^{32}P incorporation by over 90%, as previously demonstrated for other cyclic nucleotide-dependent or independent protein kinases [32—34]. These findings suggested that data obtained from this assay system truly reflected the activity of functioning protein kinases.

Plasma membrane basal protein kinase activity in normal and RSV-transformed cells

The first major question concerned the basal level of protein kinase (i.e. in the absence of exogenous cyclic nucleotides) in plasma membranes of normal and RSV-transformed chick embryo fibroblasts. A series of about 25 separate experiments was carried out to compare the membrane enzyme activity of normal and transformed cells using normal uninfected cells, cells infected with wild-type RSV, or cells infected with the mutant ts 68 incubated at either 36 or 41°C. The results of three representative experiments are given in Table I. Normal and transformed cell plasma membranes contained approximately equal amounts of basal membrane protein kinase activity. However it was found that transformed cells contained consistently higher levels, although in some cases the difference was marginal. Of the 25 separate experiments, the results of 8 studies were similar to experiment A of Table I in which higher

TABLE I

PLASMA MEMBRANE BASAL PROTEIN KINASE ACTIVITY IN NORMAL AND RSV-TRANSFORMED CHICK EMBRYO FIBROBLASTS

The in vitro phosphorylation reaction was carried out under standard conditions (10 mM $MgCl_2$) using membranes isolated from uninfected or RSV-infected cells, or from cells infected with RSV-ts 68 and incubated at either 36°C (transformed phenotype) or 41°C (normal phenotype). Experiments A, B and C represent three separate analyses on three different sets of membrane preparations. The number of pmol of phosphate incorporated was calculated from the quantity of ^{32}P present as acid-insoluble material. The experimental error varied between 9 and 13% in these experiments.

Experiment	RSV-infected cells used as membrane source (culture temperature)	Basal protein kinase activity (pmol phosphate)	Ratio (transformed/normal)
A	Uninfected (39°C)	2.17	1.1
	Wild type RSV (39°C)	2.49	—
B	Uninfected (36°C)	2.53	1.5
	ts 68 (41°C)	3.13	1.2
	ts 68 (36°C)	3.72	—
C	Uninfected (36°C)	1.69	2.4
	ts 68 (41°C)	2.76	1.5
	ts 68 (36°C)	4.01	—

amounts of protein kinase activity were found in transformed cells but the difference was within experimental error. The data from 4 other experiments were comparable to experiment C of Table I in which transformed cells were found to have about twice the activity found in normal cells. By far the most frequent result obtained (13 of 25 experiments), as demonstrated in experiment B of Table I, indicated a basal activity in transformed cells 1.2—1.6 times that of normal cells. As described in Materials and Methods, the experimental error was never more than 13%, and thus the difference was significant. It should also be pointed out that results from these studies which employed the mutant ts 68 indicated a higher basal enzyme level in membranes from cells with a transformed phenotype. Since virus production has been shown to be equivalent at the two incubation temperatures [26], differences observed were due to transformation and not to virus replication. It was therefore concluded that transformation of chick embryo fibroblasts by RSV caused a slight increase in plasma membrane basal protein kinase activity.

Effect of exogenous cyclic nucleotides on plasma membrane protein kinase activity in normal and RSV-transformed cells

Since cyclic nucleotide-dependent protein kinases may play an important regulatory role it was of importance to determine the effect of RSV transformation on this class of enzyme. Thus plasma membrane enzyme activity was measured in assays employing the addition of varying concentrations of exogenous cyclic AMP and cyclic GMP, in the presence of either 10 or 100 mM Mg^{2+}. The data obtained (Fig. 4) was expressed as the percentage stimulation obtained comparing samples incubated in the presence and absence of cyclic nucleotides. In a preliminary study (Fig. 4a) using purified beef heart protein kinase, an approximate 3-fold stimulation (i.e. 200%) in activity was obtained with cyclic AMP over a concentration range of 10^{-6} to $5 \cdot 10^{-4}$ M. In studies using plasma membranes from normal and RSV-transformed cells the effects of both cyclic AMP and cyclic GMP were tested (Fig. 4, b and c, respectively). Considering first the results obtained with membranes from normal cells, cyclic AMP stimulated protein kinase activity by about 2-fold over a range of 10^{-6} to $5 \cdot 10^{-5}$ M. In similar experiments (data not shown) using dibutyryl cyclic AMP almost identical results were obtained except that the stimulatory effect was only about 60% of that seen with cyclic AMP. Such stimulation was only detected at low (10 mM) concentrations of Mg^{2+}. At a higher, closer to optimal concentration of Mg^{2+} (100 mM) no stimulation was detected whatsoever with cyclic AMP and in fact some inhibition was detected at higher concentrations of the cyclic nucleotide. Cyclic GMP also stimulated protein kinase activity in normal chick embryo fibroblast membranes. Activity was enhanced by about 1.8-fold at 10^{-5} to 10^{-4} M cyclic GMP. Again such stimulation was only seen at low concentrations of Mg^{2+}. In marked contrast, protein kinase of plasma membranes isolated from RSV-transformed cells failed to respond to either cyclic AMP or cyclic GMP at any concentration tested, at both high and low amounts of Mg^{2+} (Fig. 4, b and c). In fact, in over 35 different experiments transformed cell membrane enzymes routinely failed to respond to cyclic nucleotides. These studies included experiments using membranes isolated from ts 68 infected cultures. Preparations from cells incubated at the restrictive temperature showed

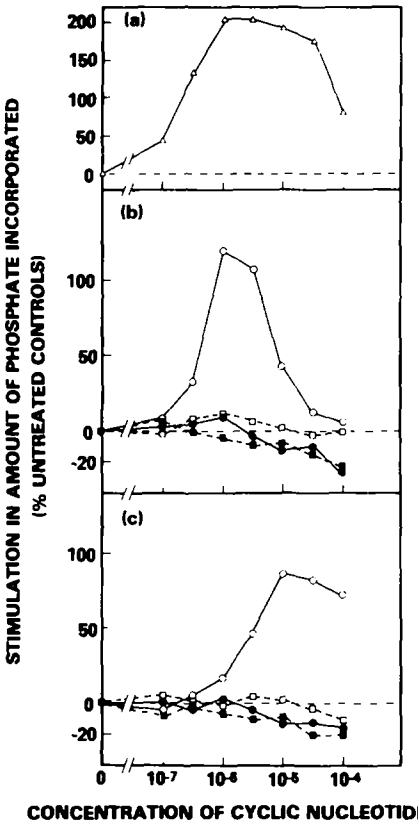

Fig. 4. Effect of cyclic nucleotides on protein kinase activity. The in vitro phosphorylation reaction was carried out under standard conditions in the presence of either 10 or 100 mM $MgCl_2$ and either in the presence or absence of cyclic AMP or cyclic GMP at the various concentrations shown in the figure. The incorporation of phosphate was determined and the results were expressed as a % stimulation of incorporation in the presence of cyclic nucleotides as compared to that obtained in their absence. The amounts of phosphate incorporated in the absence of cyclic nucleotides for samples of normal cell plasma membranes at 10 and 100 mM $MgCl_2$ were 4.31 and 6.57 pmol, respectively, and for membranes from RSV-transformed cells 5.06 and 7.62 pmol, respectively. Beef heart protein kinase (5 μg per reaction mixture) converted 21.5 pmol of phosphate to histone in the absence of cyclic AMP: (a) beef heart protein kinase (△———△) and cyclic AMP at 10 mM $MgCl_2$; (b) chick embryo fibroblast plasma membranes and cyclic AMP; (c) chick embryo fibroblast plasma membranes and cyclic GMP. Normal cell membranes incubated in the presence of 10 mM (○———○) and 100 mM (●———●) $MgCl_2$. Membranes from RSV-transformed cells incubated in the presence of 10 mM (□- - - - -□) and 100 mM (■- - - - -■) $MgCl_2$.

responses similar to normal chick fibroblasts, but cultures incubated at the permissive temperature showed no enzyme stimulation with cyclic nucleotides. Again, the studies using ts 68 indicated that the observed effects were due to transformation and not viral replication.

One further point should be made concerning cyclic nucleotide-dependent protein kinase activity. A great number of experiments have been carried out to measure the effect of cyclic nucleotides on enzyme activity. In some experiments no stimulation was seen with either cyclic AMP or cyclic GMP using membranes from normal cells. In earlier attempts however, frozen membrane preparations were used, some of which had been stored for several weeks. It

TABLE II

EFFECT OF CYCLIC NUCLEOTIDES ON PROTEIN KINASE ACTIVITY IN PLASMA MEMBRANES ISOLATED FROM NORMAL AND TRANSFORMED CELLS

The in vitro phosphorylation reaction was carried out in a series of experiments under standard conditions (10 mM $MgCl_2$) either in the presence or absence of cyclic AMP (10^{-6} M) or cyclic GMP (10^{-5} M). The number of pmol of phosphate incorporated was calculated from the quantity of ^{32}P present as acid-insoluble material. Experimental error varied from 8—12%.

Experiment	Cells used as membrane source	Protein kinase activity (pmol PO_4)				
		Basal activity	+ cyclic AMP	+ cyclic AMP / basal	+ cylic GMP	+ cylic GMP / basal
1 *	Uninfected	2.35	2.78	1.2	3.06	1.3
	RSV-infected	2.47	2.41	1.0	2.48	1.0
2 **	Uninfected	3.33	5.71	1.7	5.30	1.6
	RSV-infected	3.82	3.84	1.0	3.72	1.0
3 ***	Uninfected	4.60	8.38	1.9	6.52	1.4
	RSV-infected	5.11	5.03	1.0	5.16	1.0
4 †	Uninfected	5.20	9.36	1.8	8.23	1.6
	RSV-infected	5.68	5.59	1.0	5.63	1.0

* Plasma membranes stored frozen for 5 weeks prior to use.

** Plasma membranes stored frozen for 1 week prior to use.

*** Plasma membranes used immediately without freezing.

† Plasma membranes frozen 4 days prior to use and theophylline (8 μM) and NaF (40 μM) present.

became apparent that both basal and cyclic nucleotide-stimulated protein kinase activity was lost during prolonged storage. In addition, no inhibitor of phosphodiesterase activity was added and thus low stimulatory activity may have been due to the degradation of the cyclic nucleotides. In recent analyses (such as the one described in Fig. 4) unfrozen membrane preparations have been used, and theophylline and NaF have been added, and stimulation has been routinely observed using normal cell preparations. These points are illustrated in the results of four representative experiments shown in Table III. First, the basal protein kinase activity present in both normal and transformed cell membranes increased with decreased storage time (experiments 1 and 2) or with the use of fresh unfrozen membranes (experiment 3). Second, the stimulation of protein kinase activity in normal cell preparations by cyclic nucleotides was found consistently and at a significant level upon decreased storage time (experiments 1—3) or upon the addition of theophylline and NaF (experiment 4). And third, even under these closer to optimal experimental conditions, no stimulation was seen using membranes isolated from RSV-transformed chick embryo fibroblasts.

Thus the data from these studies showed that cyclic AMP and cyclic GMP stimulated endogenous protein kinase activity in normal but not in RSV-transformed chick embryo fibroblast plasma membranes. In addition, such stimulation was seen only using low suboptimal levels of Mg^{2+}.

Discussion

The present report represents the first attempt to investigate the effect of RSV transformation on plasma membrane protein kinases in chick embryo

fibroblasts. Experiments have been carried out using an in vitro phosphorylation system involving isolated plasma membranes from normal and transformed cells in which the membranes serve both as the source of endogenous protein substrate and protein kinase.

With respect to most reaction parameters, the plasma membrane protein kinases from both normal and RSV transformed cells were found to be similar to protein kinases from other sources (cf. refs. 10—12). However, a decline in reaction rate was observed after 5—10 min of incubation, followed by an actual loss of incorporated ^{32}P. Such was not the case using purified beef heart protein kinase with histone as substrate. This phenomenon was probably attributable to the action of ATPases and phosphoprotein phosphatases in the membrane preparations. In addition, plasma membrane protein kinases showed very different reaction kinetics as compared to the beef heart enzyme (Fig. 3) or other soluble protein kinases (cf. refs. 10—12) in response to increasing Mg^{2+} concentrations. Beef heart protein kinase possessed a farily sharp Mg^{2+} optimum at about 10 mM whereas the chick embryo fibroblast membrane-bound enzyme was stimulated by increasing amounts of Mg^{2+} up to about 100 mM. The reason for this difference is not clear. It is unlikely that Mg^{2+} was being chelated out by membrane components since purified beef heart protein kinase retained a sharp 10 mM Mg^{2+} optimum even in the presence of native or heat-denatured cell membranes (Branton, P.E., unpublished). The increased activity with high amounts of Mg^{2+} in membrane preparations could reflect a basic difference between avian and mammalian protein kinases or between cytosol and membrane-bound enzymes. The latter possibility is more likely however since similar results have been obtained with erythrocyte [35] and Chinese hamster ovary (Carlsen, S. and Ling, V., personal communication) plasma membranes and since cytosol chick embryo fibroblast protein kinases have a lower, more normal Mg^{2+} optimum (Branton, unpublished). Perhaps also high levels of Mg^{2+} affect other competing activities in the system such as ATPases or phosphatases. Studies on purified membrane protein kinases could clarify this point.

The studies presented herein have indicated that transformation of chick embryo fibroblasts by RSV consistently caused a slight increase in the basal protein kinase activity present in plasma membranes (Table I). Previous studies on partially purified whole cell protein kinases from normal and feline sarcoma virus transformed bovine cells indicated that no differences in activity occurred after transformation [36]. Also it was shown that no difference in whole cell protein kinase activity was apparent in non-growing or growth stimulated chick cells [37]. These studies were done on whole cell homogenates using exogenous substrates however and thus the results may not be directly comparable. In a more recent study in which the cell surface protein kinases of 3T3 and SV40-transformed 3T3 mouse lines were compared by the addition of exogenous radioactive ATP to intact cells, the transformed cultures were shown to have 5—10 times more activity than normal cells [38]. Since an apparent change in protein kinase activity could also result from alterations in membrane phosphoprotein phosphatases, further analyses of these enzymes and of purified plasma membrane protein kinases are necessary to determine the exact basis of this transformation-related phenomenon.

It was also demonstrated (Fig. 4) that normal cell plasma membrane protein kinase activity could be stimulated by the addition of exogenous cyclic AMP and cyclic GMP. This stimulation was lost or reduced by freezing and prolonged storage, however, under optimal conditions activity was usually increased by 1.5—3-fold. This degree of stimulation in enzyme activity was low, but that induced by cyclic AMP was quite comparable to levels found in purified membranes from other cell or tissue types (e.g. refs. 38—41). Membrane isolation may cause some loss of activity and also since the basal level of protein kinases in the absence of cyclic nucleotides is quite high, the stimulation may be partially masked. The increase of plasma membrane protein kinase activity by cyclic GMP may indicate the presence of cyclic GMP-dependent enzymes in the preparations. However, purified cyclic AMP-dependent beef heart protein kinase was also stimulated to some extent by cyclic GMP (data not shown). Thus at present it is not possible to judge whether or not such enzymes really do exist in chick embryo fibroblast plasma membranes.

The stimulation of endogenous normal cell plasma membrane protein kinase activity was markedly dependent upon the concentration of Mg^{2+}. Such activity was detected only at low suboptimal (10 mM) concentrations of this cation. At higher amounts of Mg^{2+} (40—200 mM), no stimulation whatsoever was seen (Fig. 4). Similar results have been obtained with erythrocyte membranes [35, 40]. It is possible that high levels of Mg^{2+} affect the binding of cyclic nucleotides to the regulatory subunit or that two classes of protein kinase are present in the membrane, one cyclic nucleotide-dependent with a low Mg^{2+} optimum and a second cyclic nucleotide-independent with a higher Mg^{2+} optimum. Further experiments are being carried out to investigate this problem.

Of considerable interest was the observation that transformed cells contained no detectable cyclic nucleotide-dependent protein kinase activity under any of the conditions tested. The reasons for this observation are unclear but a number of explanations are feasible. First, it might be possible to explain the present data on the basis of increased levels of cyclic nucleotide phosphodiesterases present in transformed cell preparations. A plasma membrane, low-K_m cyclic AMP phosphodiesterase has been described in chick embryo fibroblasts [42], however recent studies [43] have shown no significant differences in the level of this enzyme when comparing RSV-transformed and normal-growing chick embryo cells (as used in the present study). In any case the presence of theophylline should inhibit this enzyme. (Levels of theophylline up to 50 μM have been used with identical results). Thus this possibility seems unlikely. Second, transformed cell plasma membranes may contain higher amounts of inhibitors or modulators (cf. refs. 44—46). Third, transformation may result in the dissociation of the regulatory subunit resulting in cyclic nucleotide-independent activity, and thus perhaps in the higher basal enzyme activity found using transformed cells. Fourth, the synthesis of cyclic nucleotide-dependent membrane protein kinases may be decreased following the transformation process. And fifth, transformed cells may contain higher levels of cyclic nucleotide-dependent phosphoprotein phosphatases. In addition, differences have been reported concerning the binding of cyclic AMP by protein kinases detected in whole cell preparations from hepatoma and normal liver cells [47—48] and from normal and SV40-transformed 3T3 cells [49]. Further analyses are underway to investigate these possibilities.

It is possible that changes in membrane protein phosphorylation may play a crucial role in altering the phenotype of transformed cells and thus more detailed biochemical studies on the enzymes and substrates involved could be very significant. Studies on the pattern of plasma membrane protein phosphorylation have already been carried out and several differences have been noted between normal and RSV-transformed cells (Branton, P.E., in preparation).

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