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MODULATION OF GUANINE TRIPHOSPHATE NUCLEOTIDE BINDING TO $p21^{RAS}$ IMMUNOPRECIPITATES OF RAT LIVER PLASMA MEMBRANES BY AGENTS AFFECTING REDOX STATE

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GTP-γ-[^{35}S] and GTP-γ-[^{32}P] or GTP-α-[^{32}P] bound to plasma membranes of rat liver was immunoprecipitated using anti $p21^{V\text{-}H\text{-}ras}$. Binding was enhanced approximately 2-fold by incubation with an exogenous electron acceptor, potassium ferricyanide (but not with potassium ferrocyanide), or oxidized ubiquinone$_{10}$ and was inhibited or unaffected by incubation with reduced pyridine nucleotides (NADH or NADPH) or reduced ubiquinone$_{10}$. The results suggest a mechanism of guanine nucleotide exchange that is responsive to oxidation-reduction.

One regulatory reaction consistently implicated in modulating the rate of entry of cells into mitosis is the exchange rate of guanine nucleotide binding to low molecular weight monomeric GTP-binding proteins such as $p21^{ras}$ [1-3]. This exchange is accelerated by growth promoting factors [4-7]. The cDNAs encoding guanine nucleotide-releasing factors have recently been cloned from yeast [8] and mouse [9]. In contrast, products of several genes that lead to a slowing of mitosis are considered to slow the exchange process [10-13].

Among the factors that stimulate growth of cultured cells especially in serum-deficient media are iron compounds such as ferricyanide that are reduced at the cell surface [14, 15]. Some relationship to the oxidation-reduction state of the membrane has been suggested [16] but as yet the relationship to oxidation or reduction of specific membrane constituents is missing from our information.

In this report we measure the effect of an external electron acceptor for plasma membrane electron flow, potassium ferricyanide, as well as oxidized and reduced pyridine nucleotides and quinones on the levels of guanine nucleoside triphosphates bound to immunoprecipitates enriched in $p21^{ras}$. The results show that the addition of ferricyanide to either isolated plasma membranes from rat

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liver or p21^{ras} immunoprecipitates markedly increased GTP bound to the p21^{ras} immunoprecipitates measured both by using hydrolyzable GTP and the non-hydrolyzable analog, GTP-γ-S. Addition of pyridine nucleotides had the opposite effect of slowing or reducing the amount of GTP bound with membrane-associated p21^{ras} and was without effect on GTP bound directly to p21^{ras} immunoprecipitates.

Materials and Methods

Rat liver plasma membranes were prepared by aqueous two-phase partition as described. The 5000 X g pellet from preparation of the Golgi apparatus [17] was the starting material. The fluffy layer which contains the Golgi apparatus fraction was mixed and withdrawn with a 1 mm diameter pipette, and was excluded from the plasma membrane preparations. Cold 1 mM $NaHCO_3$ (5 ml) was added to each tube and the friable yellow-brown upper part of the pellet was resuspended with a pen-brush leaving the reddish tightly packed bottom part of the pellet undisturbed. The resuspended material was concentrated by centrifugation and was used for the two-phase separation.

The two-phase system contained 6.4% (w/w) Dextran T-500 (Pharmacia), 6.4% (w/w) poly(ethylene glycol) 3350 (Fisher), and 5 mM potassium phosphate buffer (Ph 7.2) as described [17]. Proteins were determined using the Bradford method with bovine serum albumin as standard.

The plasma membrane preparations utilized in this study have been characterized extensively based on both morphological and enzymatic criteria [17, 18]. From morphometric analysis using electron microscopy, the preparations contain 90 ± 4 percent plasma membrane. Contaminants included mitochondria (4%), endoplasmic reticulum (3%) and trace amounts of nuclear envelope, Golgi apparatus, lysosomes, peroxisomes and unidentified membranes (combined total of 3%). Based on analyses of marker enzymes, the contamination by endoplasmic reticulum was estimated to be 3%, that of mitochondria 15% and that of Golgi apparatus 1%. The yield of plasma membranes was estimated to average 18% based on recovery of enzyme markers.

Binding was determined for a reaction mixture of 20 mM Tris HCl, pH 7.5 containing 5 mM EDTA. To this mixture were added GTP-γ-[^{32}P], GTP-α-[^{32}P] or GTP-γ-[^{35}S] (0.10 to 1.0 μC_i) plus cold carrier to a final concentration of approximately 650 nM and between 40 and 300 μg of rat liver plasma membrane protein in a total volume of 50 μl. Where indicated, pyridine nucleotides or potassium ferro- or ferricyanide were added at a final concentration of 500 μM. Oxidized or borohydride-reduced ubiquinone$_{10}$ were added at a final concentration of 10 μM. The reaction was initiated by the addition of plasma membranes and was incubated for 0, 2 or 10 min at 37°C.

At the end of the incubation, 0.9 ml of a solution containing 100 mM sodium phosphate, pH 7.25, with 0.9% sodium chloride, 1.0% SDS, 1.0% (v/v) Triton X-100, 0.50% sodium deocycholate, 0.20% sodium azide and 1.0 mM sodium fluoride (PBSTDS) at 4°C was added and vortexed vigorously for 2 min at 4°. The sample was centrifuged 10 min in a microfuge and to 900 μl of the supernatant was added 15 μl of Protein G plus Agarose + 1.0 μg of the anti p21$^{V\text{-}H\text{-}ras}$ Y13-259 (both from Oncogene Science). The mixture was incubated at 4°C overnight (20 h) with gentle shaking. The sample was centrifuged and the supernatant removed. To the immunoprecipitate was added 1 ml PBSTDS and the pellet washed using microfuge centrifugation to collect the immunoprecipitate. The washing-resuspension step was repeated a total of 3 times. The final washed pellet was then resuspended and aliquots were removed for analysis of radioactivity.

For SDS-PAGE, immunocomplexes were eluted by boiling for 3 min in 30 μl SDS-electrophoresis sample buffer containing 400 μg/ml oxidized insulin A chain (Sigma) and applied to SDS-gels (8% acrylamide, 0.1% SDS) as described by Laemmli [19]. For Western blotting, the nitrocellulose sheets after transfer were washed with TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) and blocked by washing in 1 % bovine serum albumin in TBS-T for 120 min, and after with TBS-T, the secondary, alkaline phosphatase-coupled antibody in PBS was added for 30 min.

Blots were developed by incubating with 5-bromo-4-chloro-3-indolyl-phosphate [20].

Results

With both $\alpha[^{32}P]$ and $\gamma[^{32}P]$ radiolabeled guanine nucleoside triphosphates as well as GTP-γ-$[^{35}S]$, the pattern of results was similar. If isolated membranes were incubated with ferricyanide, binding was increased. With pyridine nucleotides, binding was reduced.

$p21^{ras}$-enriched immunoprecipitates from rat liver plasma membranes contained about 250 fmoles bound GTP-γ-$[^{32}P]$/mg protein in the absence of any additions. This amount was changed upon incubation with 500 μM ferricyanide to about 500 fmole GTP-γ-$[^{32}P]$/mg protein (Fig. 1). Upon incubation with 500 μM NADH the amount was essentially unchanged at 200 fmoles/mg protein. Results were similar with GTP-α-$[^{32}P]$ (not illustrated). With both GTP-γ-$[^{32}P]$ and GTP-α-$[^{32}P]$, the results with NADH were not different from untreated membranes. With GTP-γ-$[^{32}P]$ and ferricyanide, the increase in the amount of radioactivity from GTP present in $p21^{ras}$ immunoprecipitates was evident after 2 min.

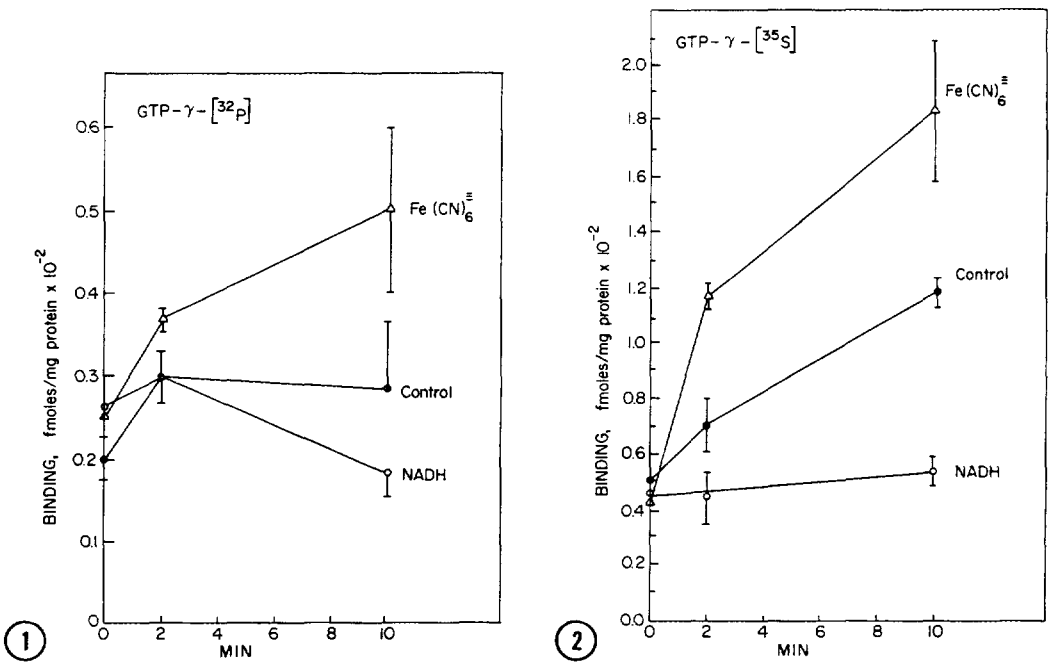

Fig.1. Binding of GTP-γ-$[^{32}P]$ to $p21^{ras}$ immunoprecipitates from rat liver plasma membranes. Compared are the effects on binding of incubation of the isolated plasma membranes with 500 μM ferricyanide or NADH compared to no additions (control). Values are means from 3 experiments plus or minus standard deviations.

Fig. 2. Binding of GTP-γ-$[^{35}S]$ to $p21^{ras}$ immunoprecipitates from rat liver plasma membranes. Compared are the effects on binding of incubation of the isolated plasma membranes with 500 μM ferricyanide or NADH compared to no additions (control). Values are from 3 experiments ± standard deviations.

Results which measure the binding of GTP-γ-[^{35}S] provided the greatest differences with the least variation (Fig. 2). Here differences were significant after both 2 min ($p < 0.05$) and 10 min ($p < 0.01$) for both ferricyanide and NADH. The binding of GTP-γ-[^{35}S] was greater than for the [^{32}P]-labeled GTP reaching ca 1,200 fmoles/mg starting plasma membrane protein with untreated membranes (Fig. 2). Binding was increased to 1,800 fmoles/mg protein by ferricyanide and was reduced to about 500 fmoles/mg protein with NADH. When initial binding of 450 fmoles/mg protein was subtracted, the increases were 750 fmoles/mg protein for the untreated membranes, 1,350 fmoles/mg protein for membranes treated with ferricyanide and 50 fmoles/mg protein for membranes in the presence of NADH.

A Western blot of the immunoprecipitates (Fig. 3) revealed a band in the 21 kDa range as the major reactive constituent. Reactive bands and proteins other than *ras* also were present in the immunoprecipitates.

The nucleotide specificity showed all pyridine nucleotides to be essentially equivalent (Table I). Both NADH and NADPH were effective in reducing GTP-γ-[^{35}S] binding as was NAD^+ and $NADP^+$, although $NADP^+$ was least effective. Potassium ferrocyanide [$K_4Fe(CN)_6$] was largely without effect compared to potassium ferricyanide [$K_3Fe(CN)_6$] (Table I).

Results similar to those with NADH and ferricyanide were obtained with oxidized or reduced ubiquinone$_{10}$ (Table II). Here ferricyanide and oxidized ubiquinone were similar in stimulating GTP-γ-[^{32}P] binding whereas both NADH and reduced ubiquinone$_{10}$ were without effect.

In other experiments, the immunoprecipitates were prepared first and then incubated with GTP-γ-[^{35}S] (Table III). The p21^{ras} immunoprecipitates bound

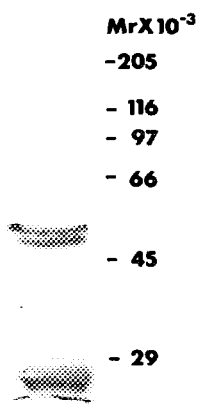

Fig. 3. Western blot analysis of immunoprecipitates. In addition to p21^{ras}, a doublet at an approximate M_r of 52 kD also was reactive.

Table I. GTP-γ-[^{35}S] binding to p21^{ras} of rat liver plasma membrane after 10 min of incubation

Addition (500 μM)	fmoles/mg plasma membrane protein ± standard deviations
None	1481 ± 345
NADH	690 ± 270
NAD^+	735 ± 100
NADPH	725 ± 200
$NADP^+$	1135 ± 130
$K_3Fe(CN)_6$	4740 ± 1030
$K_4Fe(CN)_6$	1630 ± 150

GTP-γ-[^{35}S] in the absence of the intact membrane. The amount of GTP bound by the p21^{ras} immunoprecipitates was increased by ferricyanide to approximately the same extent as when the membranes were first incubated with ferricyanide followed by p21^{ras} immunoprecipitation. However, with the isolated p21^{ras} immunoprecipitates, there was no effect of the reduced pyridine nucleotide on GTP-γ-[^{35}S] binding (Table III).

Discussion

The results show that addition of ferricyanide either to rat liver plasma membranes or to immunoprecipitates enriched in p21^{ras} increased the binding of radiolabeled guanine nucleoside triphosphates. The immunoprecipitates were obtained using an antibody to a common p21^{ras} epitope [21]. The response did not require intact membranes and was intrinsic to the p21^{ras} immunoprecipitates. The important element appeared to be electron withdrawal as oxidized (but not reduced) quinone had the same effect as ferricyanide. Potassium ferrocyanide, which is not reduced by cells [16], was without effect. These findings suggest that redox constituents may be associated closely with p21^{ras} or the p21^{ras}-like GTP-binding proteins immunoprecipitated by the antibody. Alternatively, a direct

Table II. GTP-γ-[^{32}P] binding to p21^{ras} of rat liver plasma membrane after 10 min of incubation

Addition	fmoles/mg plasma membrane protein X 10^{-2}
None	380
NADH, 500 μM	340
$Ubiquinone_{10}$ reduced, 10 μM	460
$K_3Fe(CN)_6$, 500 μM	1100
$Ubiquinone_{10}$ oxidized, 10 μM	1200

Table III. GTP-γ-[^{35}S] binding to immunoprecipitated p21^{ras} prepared from rat liver plasma membrane

Additions (500 μM)	fmoles/mg plasma membrane protein[1] 0 min	10 min
None	50 ± 8	2200 ± 180
NADH	65 ± 6	2285 ± 240
$K_3Fe(CN)_6$	143 ± 30	4200 ± 730

[1]Based on original starting plasma membrane before immunoprecipitation.

effect may be indicated, although the possibility that the precipitates contained some p21^{ras} nucleotide exchange activity and that the effect of the ferricyanide was to activate the exchanger cannot be excluded.

Pyridine nucleotides were inhibitory or without effect. The inhibitory effect of the reduced pyridine nucleotides required membranes and was not seen with isolated p21^{ras} immunoprecipitates.

There is considerable evidence (reviewed in reference [16]) that external oxidants such as ferricyanide stimulate growth. However, there has been no clear indication of how this takes place although some role of a plasma membrane redox system has been implicated. The present findings show an ability of ferricyanide or oxidized quinone to accelerate guanine nucleoside triphosphate binding to p12^{ras} immunoprecipitates of rat liver plasma membranes. This effect did not require involvement of an intact membrane. The significance of ferricyanide-accelerated guanine nucleoside triphosphate binding to p21^{ras} immunoprecipitates may rest in the central role ascribed to *ras*-related nucleoside exchange (GDP release) in the regulation of mitosis [8, 9] and may help explain why ferricyanide stimulates cell growth.

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