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Tyrosyl and Phosphatidylinositol Kinases of Human Erythrocyte Membranes

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The tyrosyl kinase and phosphatidylinositol (PI) kinase activities of human red cells have been partially purified and characterized. Although the PI kinase required detergent for solubilization, the major tyrosyl kinase of the red cell could be extracted by high salt. A very small residual activity remained associated with the membranes, however, that was solubilized with the PI kinase and copurified through an ammonium sulfate precipitation and diethylaminoethyl (DEAE) ion-exchange step gradient elution. However, the two activities were found to differ with respect to their apparent K_ms for ATP and Mg^{2+}; they showed different half-lives for temperature inactivation, possessed different relative activities in the presence of Mn^{2+} and Ca^{2+}, and were separable by elution from a DEAE-Trisacryl ion exchange column using a linear NaCl gradient. The kinetic parameters of the membrane-associated tyrosyl kinase differed from those of the salt-extracted enzyme. PI kinase was not activated by pretreatment with the tyrosyl kinase $p68^{v\text{-}ros}$ or by addition of the phosphotyrosyl phosphatase inhibitor, vanadate, to intact membranes, and was not competitively inhibited by the tyrosyl kinase substrate poly(Glu_4,Tyr). We conclude that the human red cell phosphatidylinositol and tyrosyl kinases are distinct and separable activities, and that at least two separable tyrosyl kinases are present in human erythrocytes.

Key words: tyrosine kinase, erythrocyte, vanadate, phosphatidylinositol

The proteins encoded by a number of oncogenes of retroviral origin have been shown to possess a tyrosyl-specific protein kinase activity (for recent review, see [1]). Their normal counterparts encoded by the homologous proto-oncogenes can also phosphorylate tyrosyl residues [2]. Two members of this group, $p68^{v\text{-}gag\text{-}ros}$ and $p60^{v\text{-}src}$, appeared to be associated also with a phosphatidylinositol (PI) kinase activity [3,4] and the polyoma middle T antigen has been reported to activate a PI kinase associated with cellular $p60^{src}$ [5]. The relationship between tyrosyl kinases and PI kinase activity remains obscure, however. A residual PI kinase activity present in highly purified epidermal growth factor (EGF) receptor has recently been separated

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from the intrinsic EGF binding and tyrosyl kinase activities [6], and the major tyrosyl kinase activities of two transformed cell lines, RS-1 and LSTRA, have been shown to be kinetically and immunologically distinct from the total cell PI kinase activities [7]. Moreover, we have recently distinguished $p68^{v\text{-}gag\text{-}ros}$ from its associated PI kinase by a *ros*-specific antibody that inhibits only the tyrosyl kinase activity in anti-*gag* immunoprecipitates from UR2-transformed cells [8]. Nonetheless, phosphatidylinositol phosphorylation remains of possible importance in the regulation of cell growth because it is required for the generation of inositol trisphosphate and diacylglycerol C [9]. These two products are second messengers that increase cytosolic Ca^{2+} and protein kinase C activity, respectively. Protein kinase C acts as the receptor for mitogenic tumor promoters such as phorbol esters [10], and we have proposed [3,11] that cellular transformation might in part be a consequence of the constitutive activation of protein kinase C.

The evidence against any association of PI kinases and tyrosyl kinase activities remains somewhat ambiguous, because the receptor that was studied [6] is not associated in vivo with the activation of PI turnover [11] and the kinase activities of the transformed cell lines were not purified.

To resolve this issue, we have partially purified and characterized an "authentic" PI kinase from the membranes of human erythrocytes. Erythrocytes were chosen because they contain high concentrations of the phosphorylated phosphatidylinositols [12] and have been previously shown also to possess unusually high tyrosyl kinase activity [13]. Their use also avoids the problems of interpretation associated with cells containing multiple types of membrane.

MATERIALS AND METHODS

Materials

Recently outdated packed red blood cells were provided by the American Red Cross. Triton X-100 was from Packard, ammonium sulfate was from Schwartz-Mann, DEAE-Trisacryl was from LKB, and poly(Glu_4,Tyr), angiotensin II, phospholipids, protease inhibitors, NP-40, and ATP were from Sigma. γ-^{32}P-ATP was from New England Nuclear. Cell lysates from UR2-infected chick embryo fibroblasts and anti-*gag* antiserum were provided by P. Balduzzi (Rochester, NY). Vanadate stock solutions were routinely boiled and allowed to stand for several days before use to allow decomposition of polymeric species.

Purification

Ghosts were prepared from human red cells by lysis in hypotonic solution (5 mM Na phosphate, ph 8.0, 0.5 mM ethylenediamine tetraacetic acid [EDTA], 0.5 mM EGTA, 1 mM dithiothrietol [DTT], plus 1 μg/ml pepstatin and 20 μg/ml phenylmethyl sulfonyl fluoride as protease inhibitors) and washed by repeated centrifugation and suspension [14]. The resulting membranes were solubilized by incubation with buffer A (10 mM Na phosphate, pH 7.4, containing 0.5 mM EDTA, 1 mM DTT and 1% Triton X-100). Insoluble cytoskeletal elements were removed by centrifugation. Sodium phosphate (pH 7.4) was then added to bring the concentration to 50 mM, followed by addition of solid ammonium sulfate to 2 M. The suspension was centrifuged (20,000g for 30 min), and the protein-detergent complex, which had floated to the surface, was collected by removal of the subnatant. The precipitate was

dissolved and dialysed against buffer B (as buffer A but with 0.05% detergent). DEAE-Trisacryl, previously equilibrated with buffer B, was stirred into the dialyzed material and incubated for 10 min. The resin was washed with three volumes of buffer B and a single-step elution was performed using three volumes of buffer B plus 200 mM NaCl. The eluant was concentrated by ultrafiltration with an Amicon YM100 filter (100,000 dalton cut-off) and stored at −80°C after freezing in liquid nitrogen.

Assays

PI kinase activity was determined by a modification of the procedure of Buckley [15], using 1 mM γ-^{32}P-ATP (10 μCi/μmol) in 25 mM Hepes-NaOH, pH 7.4, 10 mM $MgCl_2$, 10 mM dithiothrietol, 0.5% Triton X-100 plus 0.5 mM phosphatidylinositol, and terminated by addition of 5% (w/v) trichloracetic acid (0°C) and 100 μg of carrier bovine serum albumin. The precipitate was centrifuged, and the pellet was rinsed with 0.5 ml of ice-cold water. The pellet was dissolved by addition of 200 μl of chloroform/methanol/HCl (2:1:0.03 N). A volume of 50 μl was spotted onto a Baker Si250 thin layer chromatography (t.l.c.) plate and developed in chloroform/methanol/ammonium hydroxide (9:7:4 N). The ^{32}P-phosphatidylinositol-4-phosphate was located by autoradiography, scraped off the plate, and counted for ^{32}P.

Tyrosyl kinase activity was routinely assayed using as substrate 1 mg of poly(Glu_4,Tyr) per ml [16] in 25 mM Hepes-NaOH, pH 7.4, 10 mM $MgCl_2$. The reaction was terminated by addition of electrophoresis sample buffer. An aliquot was electrophoresed on a 10% sodium dodecyl sulfate (SDS) acrylamide gel, stained with Coomassie blue, dried, and either autoradiographed or directly counted by excising each lane between molecular weight markers of 27K and 55K daltons and counting for ^{32}P.

Protein concentrations were determined by a modification of the method of Lowry [17].

RESULTS

Assay and Purification of Phosphatidylinositol and Tyrosyl Kinase Activities

The PI kinase assay used in the present study proved considerably more rapid than those described previously [eg, 12,15] because of the absence of two-phase extraction steps common to earlier methods. Carrier protein was necessary to ensure complete precipitation of the phospholipid. Using solubilized membranes assayed under standard conditions, the only ^{32}P-labeled spots detectable above the origin on t.l.c. were phosphatidylinositol-4-phosphate and lysophosphatidylinositol-4-phosphate, as judged by comparison with authentic marker lipids. The time course for phosphorylation was linear for at least 60 min using solubilized material. As reported previously [18], red cell membranes also possess tyrosyl-specific protein kinase activity. Under standard assay conditions the reaction was linear for 5 min when using membranes and for more than 30 min when using solubilized material.

Most of the red cell tyrosyl kinase activity was found to be membrane bound, but was released by treatment with a high salt concentration (Table I). The $K_{1/2}$ for release was about 150 mM NaCl (Fig. 1). The soluble tyrosyl kinase activity was stable for several months after freezing in liquid nitrogen and storage at −80°C. The K_m for ATP was 9.7 μM. Addition of 0.5% Triton X-100 to the assay buffer increased the K_m slightly, to about 20 μM. The PI kinase activity, on the other hand, together

TABLE I. Partial Purification of Phosphatidylinositol and Tyrosyl Kinase Activities From Human Erythrocytes*

Purification step	Total protein (mg)	PI kinase activity (nmol mg^{-1} min^{-1})	Fold purification	Percent yield	Tyrosyl kinase activity (nmol mg^{-1} min^{-1})
Total cell lysate	33903	0.02	1	100	0.016
Membranes	661	0.66	44	64.3	0.87
Salt wash (250 mM NaCl)	N.D.	<0.01	—	—	0.85
Triton X-100 supernatant	366	1.27	64	68.6	0.004
Ammonium sulfate precipitation	33.3	3.73	187	18.3	0.026
DEAE step-elution	3.6	22.2	1110	11.8	0.280

*Activities were purified from one unit of recently-outdated human red cells, as described in "Materials and Methods." PI kinase was assayed using 1 mM ATP (γ-^{22}-P-ATP, 50 μCi/μmol) and 0.5 mM PI; tyrosol kinase was assayed using 0.2 mM ATP (γ-^{32}-P-ATP, 250 μCi/μmol) and 0.2 mg/ml poly (Glu_4, Tyr) as substrate. This purification was typical of six similar preparations, providing a mean PI kinase activity after the DEAE step of 23.8 ± 5.6 nmol/mg/min (± 1 SD).

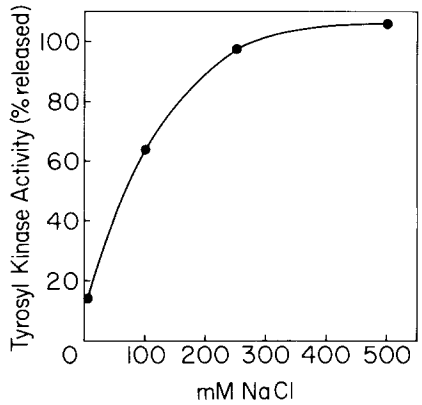

Fig. 1. NaCl-dependent elution of tyrosyl kinase from human red cell membranes. Membranes were diluted with NaCl to the concentrations indicated, incubated on ice for 5 min, then centrifuged in a microfuge for 10 min (4°C). Supernatants were adjusted to the same final NaCl concentration and assayed as described in "Materials and Methods."

with a small fraction of the tyrosyl kinase activity, required the presence of a nonionic detergent such as Triton X-100 for solubilization. These two activities were precipitated by 40% ammonium sulfate (w/v) and were retained by a DEAE anion exchanger at pH 7.8 at low ionic strength and eluted together by 200 mM NaCl. The salt-extractable tyrosyl kinase, however, was not retained by DEAE cellulose at pH 7.8 (data not shown). Typical purification results are shown in Table I. In six similar purifications, the mean PI kinase activity of the DEAE eluant was 23.8 ± 5.6 nmol/mg/min, representing about a 1,000-fold purification from the initial cell lysate. The membrane tyrosyl kinase activity was about two orders of magnitude lower than this,

under standard assay conditions. No PI-phosphate kinase or diacylglycerol kinase activity was detectable.

The material obtained by batch elution from the DEAE-Trisacryl was used for further kinetic analysis.

Characterization of Erythrocyte PI and Tyrosyl Kinase Activities (Table II)

To determine whether a single enzyme is responsible for the two detergent-solubilized kinase activities found in the erythrocyte membrane, we examined their kinetic characteristics with respect to ATP and M^{2+} concentration and to thermal inactivation. The effect of varying ATP is shown in Figure 2. Curves were fit to the data by nonlinear least-squares using the standard Mento-Michaelis equation. The apparent K_ms for PI and tyrosyl kinase were 0.14 mM and 0.34 mM, respectively. That these values are significantly different can be judged from the dashed curves of Figure 2, generated by reversing the respective K_m values. The K_m for the detergent-solubilized tyrosyl kinase is also significantly different from that of the salt-extracted enzyme (0.02 mM) when measured under very similar conditions.

The effect of varying Mg^{2+} concentration is shown in Figure 3. Free Mg^{2+} concentrations were calculated assuming a pKa for ATP of 6.97 and a pK($Mg \cdot ATP^{4-}$) of 4.49. Again the apparent K_ms differ significantly, being 0.63 mM and 7.2 mM for the PI and tyrosyl kinases, respectively. We also found that addition of 10 mM Ca^{2+} inhibited the PI kinase activity by 98% but the tyrosyl kinase activity by only 75%. Ca^{2+} alone was unable to support substrate phosphorylation by either activity. Mn^{2+} (2 mM) supported 30% of the PI kinase activity observed with 10 mM Mg^{2+}, possibly as a result of contaminating Mg^{2+}. Under the same conditions, tyrosyl kinase activity was undetectably low.

Inactivation rates at two different temperatures were also compared. At 37°C the rate of PI kinase inactivation ($t_{1/2}$ of 4.8 hr) was significantly slower than that of tyrosyl kinase ($t_{1/2}$ of 2.8 hr). At 50°C, in the absence of the phospholipid, inactivation was rapid and apparently first order, with $t_{1/2}$s of 2.5 min and 5.1 min for the PI and tyrosyl kinase, respectively.

To determine if the substrates of the two kinases were competitive, PI kinase activity was examined in the presence of poly(Glu_4,Tyr) at 0–4 mg/ml, plus an

TABLE II. Comparison of Phosphatidylinositol and Tyrosyl Kinase Activities of Human Erythrocyte Membranes*

Enzyme	K_m for ATP	K_m for Mg^{2+}	Preferred cation	$T_{1/2}$ for inactivation 37°C	$T_{1/2}$ for inactivation 50°C
Salt-extracted tyrosyl kinase	0.01-0.02 mM[a]	10mM[c]	Mn^{2+}[b]	N.D.	N.D.
Detergent-solubilized tyrosyl kinase	0.34 mM	7.2 mM	Mg^{2+}	2.8 hr	5.1 min
Detergent-solubilized phosphatidylinositol kinase	0.14 mM	0.63 mM	Mg^{2+}	4.8 hr	2.5 min

*Parameters were determined as described in "Materials and Methods."

[a]The apparent K_m was increased by inclusion of 0.5% Triton X-100 in the assay buffer. Mohamed and Steck [19] reported a K_m of 2.5 μM in the absence of detergent.

[b]Mohamed and Steck [19].

[c]Phan-Dinh-Tuy et al. [18].

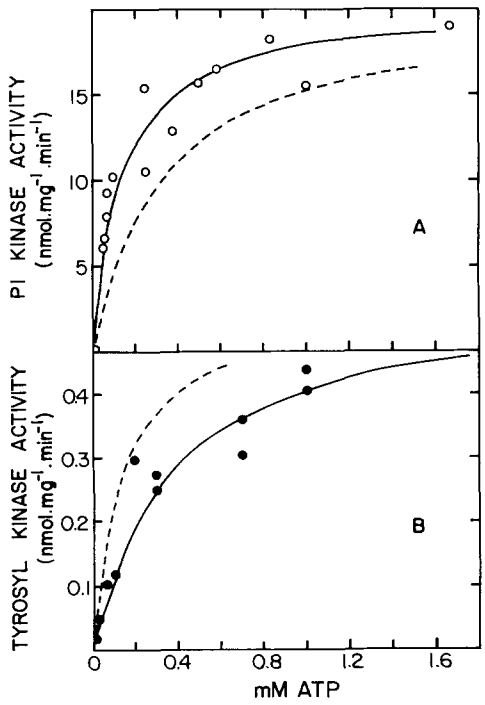

Fig. 2. Effect of varying ATP concentrations on PI (**A**) and tyrosyl (**B**) kinase activities. Assays were performed on DEAE-purified material (Table I) as described in "Materials and Methods." Curves were fit to the data by an iterative nonlinear least-squares procedure, using the standard Menton-Michaelis equation. The best-fit parameters were: (A) K_m = 0.14 mM, V_{max} = 0.49 mol mg^{-1} min^{-1}. Dashed lines show fits using the same values for V_{max} but opposite values for the K_m (0.34 in A and 0.14 in B).

additional 5 mM $MgCl_2$ to avoid artifactual effects of complexation by the polyanionic substrate. No inhibition of the PI phosphorylation was detected (data not shown).

These results all indicate that the human red cell membrane contains distinct tyrosyl and PI kinases. Moreover, the kinetic data for the detergent-solubilized tyrosyl kinase are significantly different from those recently reported for the salt-extractable enzyme [18,19], suggesting that at least two different tyrosyl kinases are present in red cell membranes.

Separation of Kinase Activities by Ion-Exchange Chromatography

In view of the above results, we further examined the copurification of the two detergent-solubilized kinase activities by using a DEAE-Trisacryl column loaded with protein from the ammonium sulfate precipitation step and eluted with a linear NaCl gradient. Results are in Figure 4. As expected, both enzyme activities were retained by the column and eluted at 100–200 mM NaCl. However, the PI kinase eluted three fractions ahead of the tyrosyl kinase (at about 130 and 170 mM NaCl, respectively). Only one major peak of each kinase was detected. A similar separation was obtained on two different preparations. Therefore, the two kinase activities represent different enzymes with nonoverlapping substrate specificities.

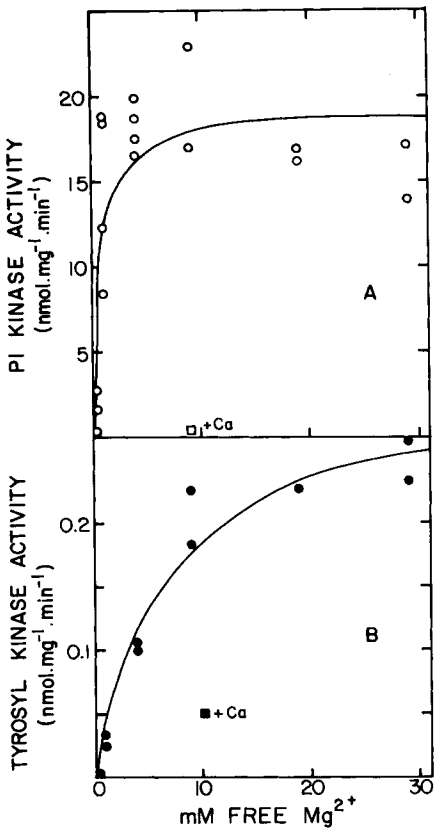

Fig. 3. Effect of varying Mg^{2+} concentration on PI (**A**) and tyrosyl (**B**) kinase activities. In one instance 10 mM $CaCl_2$ was also added (□, ■). Curves were fit to the data using an iterative nonlinear least-squares procedure with the standard Menton-Michaelis equation. The $K_{1/2}$s obtained were 0.63 (A) and 7.2 mM (B).

Effect of Tyrosyl Kinases on PI Kinase Activity

To investigate whether tyrosyl phosphorylation of the PI kinase might activate the enzyme, we preincubated the partially purified PI kinase in the presence of 100 μM cold ATP at 30°C for 30 min with $p68^{v\text{-}gag\text{-}ros}$, which had been immunoprecipitated from UR2-infected chick embryo fibroblasts by anti-gag antiserum as in [3]. The immunoprecipitate was then removed by centrifugation, and the supernatant PI kinase activity was compared with a mock-treated control. No significant difference in phospholipid phosphorylation was detected between the two samples. The $p68^{v\text{-}gag\text{-}ros}$ was able to phosphorylate poly(Glu_4,Tyr), but no phosphorylated bands were detected when a sample of the preincubated PI kinase was analysed by electrophoresis on a 10% SDS gel. These results suggest that the PI kinase is not a good substrate for the $p68^{v\text{-}gag\text{-}ros}$ tyrosyl kinase.

It remained possible, however, that the PI kinase might be phosphorylated and activated specifically by the erythrocyte tyrosyl kinase under certain conditions. We have therefore studied the effect of added vanadate on PI and tyrosyl phosphorylations in intact erythrocyte membranes. Vanadate is a potent inhibitor of phosphotyrosyl

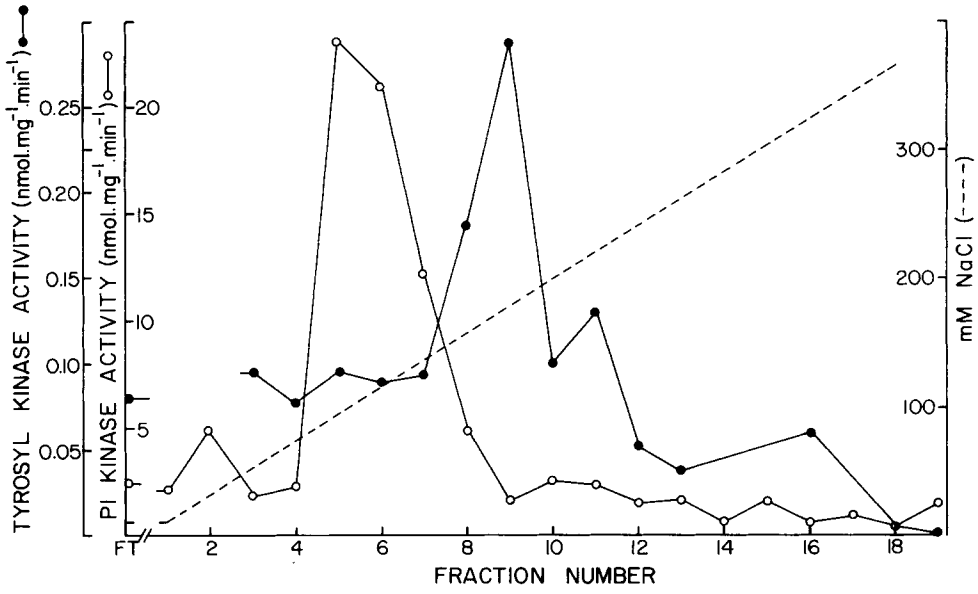

Fig. 4. Separation of PI (○) and tyrosyl kinase (●) activities by DEAE-Trisacryl ion-exchange chromatography. The column was equilibrated with starting buffer (10 mM Na phosphate pH 7.4, 1 mM DTT, 0.5 mM EDTA, 0.05% NP-40) and loaded with ammonium sulfate-precipitated material dialyzed extensively against starting buffer. The column was then eluted with a 0–500 mM linear NaCl gradient in starting buffer. Fractions were assayed for PI, tyrosyl kinase activities, and protein concentration as described in "Materials and Methods."

phosphatases [20] and might be expected to increase the level of tyrosyl phosphorylation of proteins in the membranes. As can be seen from Figure 5A, addition of 20 μM ammonium vanadate more than doubled the incorporation of ^{32}P into poly(Glu_4,Tyr) and decreased by half the release of ^{32}P-phosphate catalyzed by the membranes (Fig. 5B). However, vanadate had little effect on the initial rate of phosphate incorporation into PI (Fig. 5C), suggesting that increasing the phosphotyrosyl content of the membranes does not significantly activate the PI kinase. A small and variable stimulation of the total level of PI-^{32}P-phosphate formation was frequently observed in these experiments, however. This effect was not a result of an inhibition of PI-phosphate phosphatase activity, which was very low in these membrane preparations and was unaffected by vanadate (rate of PI-^{32}P breakdown was $0.24\% \cdot min^{-1}$ at 37°C). Nor was it likely to have been a result of a variable preexisting phosphorylation state of the PI kinase, since pretreatment with calf intestinal alkaline phosphatase, which can efficiently dephosphorylate phosphotyrosyl residues, did not significantly effect the PI kinase activity (data not shown). The most likely explanation is that the small stimulation was caused by inhibition of membrane ATPases by the vanadate, resulting in the maintenance of a slightly higher ATP concentration over the course of the experiment (Fig. 5B). Whatever the cause, it appears unlikely that tyrosyl phosphorylation can activate the red cell PI kinase.

DISCUSSION

We have partially purified the phosphatidylinositol and tyrosyl kinase activities present in human erythrocyte membranes and have shown them to be kinetically

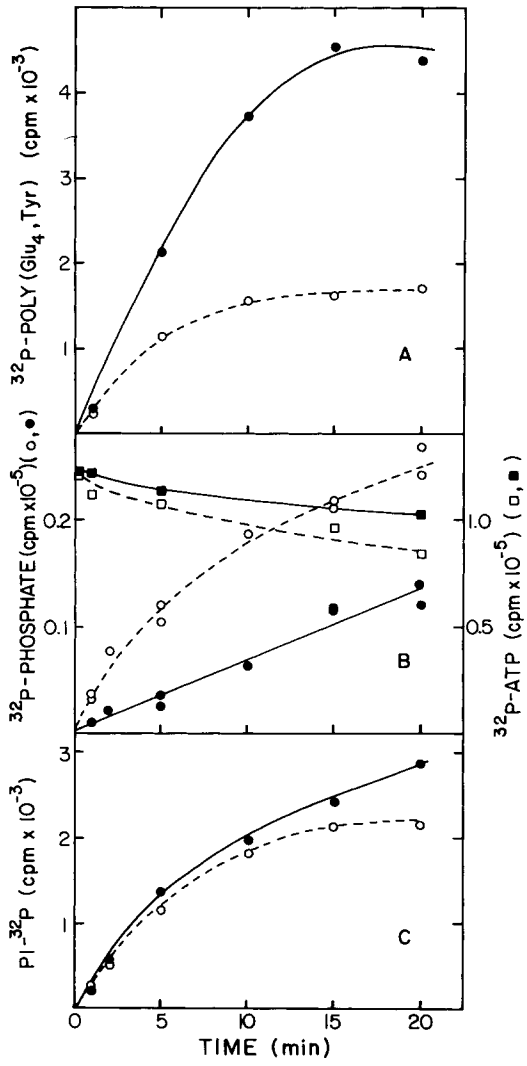

Fig. 5. Effect of vanadate on tyrosyl kinase activity (**A**), phosphate production and ATP utilization (**B**), and PI kinase activity (**C**) by erythrocyte membranes. Membranes were prepared as in [14]. Incubations were performed at 37°C in the presence (●, ■) or absence (○, □) of 20 μM sodium vanadate. Tyrosyl kinase activity was measured using poly(Glu$_4$,Tyr) as substrate, and PI kinase activity was measured using endogenous PI as substrate. Assays were performed in the absence of detergent. Remaining ^{32}P-phosphate was precipitated after stopping the reaction, as in [21]. Precipitates were centrifuged, and the pellet was washed three times in the precipitating solution and counted for ^{32}P (○, ●). A sample of the first supernatant, containing the γ-^{32}P-ATP, was spotted onto a PEI-cellulose t.l.c. plate and chromatographed using 0.75 M Tris, 0.45 HCl, and 0.5 M LiCl as solvent, as in [22]. Following autoradiography the ATP spot was cut out and counted for ^{32}P (□, ■). Mean recovery of counts was 86.6 ± 8.3% (ATP + phosphate).

distinct and chromatographically separable activities. We have found that at least two tyrosyl kinases are present in the membranes, the principal one of which is a peripheral membrane protein released by high salt, as has recently been reported also by others [19], plus a minor component that copurifies through several steps with the PI kinase. Manipulations to try and phosphorylate the PI kinase did not increase its activity. We conclude that the kinase activities reside on separate polypeptides and that they do not interact in any obvious fashion. Nonetheless the two detergent-solubilized enzymes bear some similarities to one another: they both act as integral membrane proteins and copurify through a number of steps; and they show different but similar rates of heat inactivation and similar divalent cation preferences. Moreover, McDonald et al [7] have recently reported that a monoclonal antibody against $p60^{src}$ appears to be able to recognize a PI kinase from certain cell types. Whether the two classes of kinase show significant homology at the sequence level, however, must await the complete purification of the PI kinase.

The membrane-bound, detergent-solubilized tyrosyl kinase appears to be distinct from the salt-extractable tyrosyl kinase described in this report and reported earlier by Mohamed and Steck [19]. The kinetic properties and characteristics on purification of a tyrosyl kinase from human red cells described by Pha-Dinh-Tuy et al [18] make it likely that it is identical with the salt-extracted enzyme even though detergent was used for the initial solubilization from membranes. It is likely, therefore, that there are two distinct tyrosyl kinases in human red cells. The $K_{1/2}$s of these two tyrosyl kinase activities for Mg^{2+} are similar, but the salt-extracted kinase appears to prefer Mn^{2+} to Mg^{2+} under the assay conditions of Mohamed and Steck [19], and the apparent K_m for ATP is significantly different (20 μM as compared to 340 μM for the detergent-solubilized enzyme in the present study). Moreover, the salt-extracted enzyme appears to be positively charged at physiological pH since it binds to a cation exchanger [18] and to the anionic portion of band 3 [19], whereas the detergent-solubilized kinase is not retained by cation exchangers (unpublished observation) and requires approximately 150 mM NaCl for elution from DEAE. The functions of these kinases in the enucleated red cell and their relationship to other known tyrosyl kinases associated with growth factor receptors and oncogene proteins remain obscure.

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