PROTEIN KINASE ACTIVITY ASSOCIATED WITH
THE SURFACE OF GUINEA PIG MACROPHAGES*

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Macrophages play multiple roles in host defense, e.g. in the induction of the immune response (1), in phagocytosis (2), and in killing bacteria and tumor cells (3). In many of its biological functions the role of the surface membrane is prominent. Macrophages can undergo extensive alterations in morphology and functions; particularly noteworthy is the increase of defense properties associated with bacterial infections (4).

For a variety of enzymes, functional alterations have been shown to be based on phosphorylation reactions (5). This paper documents the identification of a protein kinase associated with the outer surface of guinea pig peritoneal macrophages. The surface-associated protein kinase is shown to transfer γ-phosphate of ATP to certain endogenous macrophage proteins and also to added acceptor protein.

Materials and Methods

Harvest of Macrophages. Hartley guinea pigs were injected intraperitonally with 30 ml 1% sodium caseinate (6). Peritoneal exudate cells were collected 5 days later and washed twice by pelleting at 300 g X 5 min using cold Hanks’ balanced salt solution (HBSS). In seven experiments the leukocytes consisted of 96 ± 1% macrophages (Wright’s stain) and 4 ± 1% polymorphonuclear leukocytes. Erythrocyte contamination averaged 6 ± 1% of the total. As judged by trypan blue exclusion, the preparations were ≥98% intact.

Phosphorylation of Macrophages. Four to six X 10⁷ macrophages were suspended to 700 μl in HBSS at room temperature with additives included in a portion of this media. The reaction was started by addition of 700 μl of 150 μM [32P]ATP in HBSS at room temperature and stopped after 1 min with 1 ml hot 4% sodium dodecyl sulfate (SDS), 4% mercaptoethanol, 2 mM phenylmethyl sulfonylfluoride. In control experiments, macrophage monolayers were prepared by incubating 3 X 10⁸ macrophages in 1 ml HBSS in 30 X 10 mm Falcon dishes at 37°C for 40 min and washed by immersing the dishes in three beakers of HBSS at room temperature. Monolayer macrophages were immediately reacted with 75 μM [32P]ATP for 1 min at room temperature.

SDS-Polyacrylamide Gel Electrophoresis. Electrophoresis was according to the method of Laemmli (7) as described.¹ Approximately 200 μg protein from 1–1.5 X 10⁸ macrophages were applied per lane. Molecular weight markers were: myosin (200,000), β-galactosidase (130,000), phosphorylase a (94,000), albumin (68,000), creatine kinase (40,000), and carbonic anhydrase (29,000). After destaining in 40% methanol and in 7% acetic acid, the gels were dried and placed in contact with Kodak No-Screen NS-5T film at -70°C for 3–12 days.

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¹ L. W. Heck et al. 1978. DFP-sensitive polypeptides of the guinea pig peritoneal macrophage. Biochem. Biophys. Res. Commun. 83:1576.
Quantitation of Radioactive Proteins. Samples fractionated on SDS gels were dried. The lanes were cut into slices of 1-3 mm, rehydrated with 400 μl 5% SDS, and counted using toluene-Liquifluor-BioSolv (86:4:10).

Quantitation of [32P]ATP Hydrolyzed. After reaction of macrophages with [32P]ATP, samples were centrifuged for 30 s at 2500 g. Supernates were chromatographed on polyethyleneimine-impregnated cellulose with 1 M LiCl. The chromatogram was cut and the resin counted as described above.

HBSS was from Microbiological Associates, Walkersville, Md. AG1-X8 resin was from Bio-Rad Laboratories, Richmond, Calif., and polyethyleneimine-impregnated plates from Brinkmann Instruments, Inc., Westbury, N.Y. [32P]phosphoric acid and Liquifluor scintillation solution were from New England Nuclear Corp., Boston, Mass., and Bio-Solv Solubilizer BBS-3 from Beckman Instruments, Inc., Fullerton, Calif. Glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and glyceraldehyde-3-phosphate were from Boehringer-Mannheim Biochemicals, Indianapolis, Ind. Prostaglandin E1 was donated by Dr. John Pike, The Upjohn Co., Kalamazoo, Mich. Cyclic AMP, ATP, and histone (Type II-AS) were from Sigma Chemical Co., St. Louis, Mo. Sodium caseinate (practical grade) was from Eastman-Kodak Co., Rochester, N.Y., and pronase from Calbiochem, San Diego, Calif.

[γ-32P]ATP prepared by the method of Glynn and Chappell (8) was purified on AG1-X8 chloride columns. Purity was monitored by chromatography on polyethyleneimine-impregnated cellulose. The product was greater than 99.5% [32P]ATP; sp act ranged from 1 to 3 Ci/mmol.

Results

Freshly isolated intact peritoneal macrophage preparations incubated with [γ-32P]-ATP incorporate phosphate into cellular components. To characterize the phosphorylated components, cells were solubilized and fractionated on polyacrylamide gels containing SDS. Fig. 1 shows the Coomassie Blue-staining pattern of total solubilized macrophage proteins and an autoradiograph of the same sample. Major phosphorylated bands were observed with apparent mol wt of 68,000, 54,000, 51,000, and 43,000. Less intensely labeled bands include components at 92,000, 87,000, 75,000, and 64,000. The dark band at the front which migrates faster than the peptide front presumably does not represent protein-bound phosphate. Based on scintillation counting of gel slices, the 68,000 and 43,000 components each incorporate 0.5 pmol 32P per mg of cell protein.

Nature of the Phosphorylated Products. Macrophage preparations after reaction with [32P]ATP were solubilized with SDS and digested with pronase. This treatment removed the Coomassie Blue-staining material and all the 32P-label (not shown), indicating that the labeled components are proteins. Also, macrophages reacted with ATP labeled in the adenosine moiety with 3H and fractionated on SDS gels showed no radiolabeled components when subjected to fluorography (9). These experiments show that the phosphorylated components are proteins containing covalently-bound phosphate.

Characteristics of the Phosphorylation Reaction. Macrophages solubilized with SDS before incubation with [32P]ATP showed no radioactive components, demonstrating that the 32P-labeled polypeptides do not form in denatured preparations. The extent of phosphorylation of the endogenous proteins was found to increase as the [32P]ATP concentration was raised over the range 7-75 μM. The same protein components were phosphorylated at 4°C and at room temperature; the extent of phosphorylation was greater at room temperature (not shown). Phosphorylation was maximum for all components except the 68,000 dalton polypeptide by 30 s to 1 min at room temperature (Fig. 2).
Fig. 1. Coomassie Blue-staining pattern and autoradiograph of macrophages reacted with [γ-32P]ATP, solubilized with SDS, and fractionated on polyacrylamide gels. On the left is the Coomassie Blue-staining pattern of total macrophage proteins with the positions of the marker proteins (molecular weight X 10^{-3}) indicated on the far left. On the right is the autoradiograph. Arrows indicate phosphorylated bands with apparent mol wt of 68,000, 54,000, 51,000, and 43,000.

Fig. 2. Time-course of phosphorylation. Autoradiograph is of SDS-gels of macrophages reacted with 75 μM [32P]ATP at room temperature for a, 0.5 min; b, 2 min; c, 5 min; and d, 10 min. For orientation, arrows indicate the same components as in Fig. 1.

The extent of hydrolysis of 75 μM [32P]ATP by macrophages averaged 61 ± 1% at 1 min and 89% after 2 min. This approximate rate was predictable from the known activity of macrophage ecto-ATPase (10). Rapid hydrolysis of ATP could explain the early termination of phosphorylation shown in Fig. 2.

It is of note that prostaglandin E1 at 10^{-5} M added immediately before the reaction did not alter the extent nor the pattern of protein phosphorylation. Cyclic AMP at 10^{-6} and 10^{-5} M were also without effect (not shown).

Exudate macrophage preparations contain a few percent erythrocytes. No phosphorylated components were observed when washed guinea pig erythrocytes were incubated with [32P]ATP using conditions established for macrophages. Similar attempts to react [32P]ATP with guinea pig serum (1.3 mg/ml) showed no labeled components, suggesting that the protein kinase is associated with the macrophage.

Possible Contribution of Broken Cells. To answer this question, [32P]ATP was reacted with monolayer macrophages. In these experiments, freshly-prepared macrophage monolayers were vigorously washed to remove nonadherent cells and broken cell components immediately before reaction with [32P]ATP. The same pattern of protein phosphorylation was observed (not shown).

For comparison, intracellular proteins were also phosphorylated by incubating intact macrophages with low concentrations of inorganic [32P]phosphate for long times (40 μM for 30 min at 37°C). 32P was incorporated into a wide spectrum of
proteins with an electrophoretic pattern grossly dissimilar from intact cells phosphorylated with \[^{32}P\]ATP.

**Extracellular vs. Intracellular Location of the Endogenous Phosphorylation Reaction.** As mentioned, the same macrophage proteins were phosphorylated by \[^{32}P\]ATP at room temperature and at 4°C, making a role for pinocytosis of ATP unlikely. However, ATP hydrolysis followed by uptake of inorganic \[^{32}P\]phosphate was a possibility. The phosphorylation patterns for macrophages reacted with 75 μM \[^{32}P\]ATP in the absence and presence of 750 μM nonradioactive inorganic phosphate were found to be identical (Fig. 3, lanes a and b) which shows that nonradioactive inorganic phosphate does not compete. Also, when \[^{32}P\]ATP was replaced by inorganic \[^{32}P\]phosphate, only a small amount of radioactivity was incorporated into a single component (Fig. 3, lane d), i.e. inorganic \[^{32}P\]phosphate does not serve as phosphate donor. These experiments indicate that the \[^{32}P\]-phosphate moiety of ATP is transferred directly and extracellularly into macrophage proteins.

**Phosphorylation of Added Accepter Protein.** Phosphorylation of added acceptor protein by intact cells would further demonstrate a surface location for the protein kinase. Histone was added to macrophages and the mixture reacted with \[^{32}P\]ATP. Fig. 4, lanes a and c show a major phosphorylated component which comigrates with histone and is absent in control macrophages (lane b). When the cells were removed by centrifugation, the \(^{32}P\)-labeled histone remained in the cell-free supernate (lane d) showing that added histone is phosphorylated extracellularly by a macrophage protein kinase.

**Is the Protein Kinase Released from the Cell During the Reaction?** Macrophages were incubated under phosphorylation conditions but without ATP and then separated into a pellet (cells) and a cell-free supernate. Using phosphorylation of added histone as an assay, the protein kinase activity of the cell-free supernate was 6 and 3% (two experiments). 94 and 97% of the protein kinase activity was found associated with the cell pellet. Identical results were obtained when ATP (75 μM, nonradioactive) was included in the first incubation. This shows that protein kinase is not released from the macrophage during the reaction. It indicates that an enzyme associated with the outer surface of macrophages is responsible for the phosphorylation of added histone.

**Discussion**

Protein kinase activity has been detected associated with the outer surface of guinea pig macrophages. When intact cells are incubated with \[^{32}P\]ATP, the \(^{32}P\)-phosphate moiety (but not the adenosine moiety) is rapidly transferred to specific macrophage surface proteins. Nonradioactive inorganic phosphate does not compete with \[^{32}P\]ATP, nor does inorganic \[^{32}P\]phosphate serve as substrate, demonstrating that transfer of phosphate to protein is both direct and extracellular. The surface protein kinase also phosphorylates added acceptor protein (histone). Protein kinase activity is shown to be firmly associated with the cell surface.

Five points indicate that the protein kinase is located on the cell surface. (a) ATP does not enter the cell under the conditions used (1 min at room temperature). (b) Similar phosphorylation takes place at 4°C where pinocytosis does not occur. (c) Inorganic phosphate does not substitute for ATP. (d) Added acceptor protein is also phosphorylated. (e) Contribution of soluble protein kinase (from broken cells) is unlikely since minimal activity was found in the cell-free supernatant.
FIG. 3. Role of inorganic phosphate in the phosphorylation of macrophages. Autoradiographs are of SDS-gels of macrophages reacted with a, 75 μM [32P]ATP; b, 75 μM [32P]ATP + 750 μM nonradioactive inorganic phosphate; c, same as b, another experiment; d, 750 μM inorganic [32P]-phosphate. c and d each contain 5 μCi 32P per incubation. Arrows are as in Fig. 1.

FIG. 4. Phosphorylation of added histone by macrophages. Autoradiographs from two experiments are shown. a and c, 6 × 10⁷ macrophages were reacted with [32P]ATP and 140 μg histone for 1 min; then cells + media were solubilized and electrophoresed. b, control without histone. d, the cell-free supernate of the preparation in c. Arrows indicate the position of histone. Incubation of histone with [32P]ATP (without macrophages) showed no phosphorylated components (not shown).

Thus, the surface protein kinase of the macrophage joins a growing list of ectoenzymes, membrane enzymes with their active site facing the extracellular medium (11). Several of the macrophage ecto-enzymes have specificities for nucleotides; these include 5'AMPase (12), ATPase (11), and alkaline phosphodiesterase (13) in addition to the protein kinase.

A few reports describe protein phosphorylation in intact cells due to extracellular ATP. Intact glial cells transfer γ-phosphate of ATP to histone added as an external acceptor protein (14). An endogenous protein kinase has been described in the outer plasma membrane of 3T3 cells which transfers γ-phosphate of external ATP to multiple cell surface proteins (15). Unlike the macrophage, the extent of surface phosphorylation in the 3T3 cell is not controlled by an ecto-ATPase.

The physiological role of the macrophage surface protein kinase is at present not known. Phosphorylation might alter the activity of macrophage surface components or, alternatively, could affect proteins on the surface of other cells.

Summary

Protein kinase activity has been detected associated with the outer surface of guinea pig peritoneal macrophages. Macrophages incubated with [γ-32P]ATP incorporated 32P-phosphate into cell-associated proteins. Inorganic phosphate did not compete, nor could inorganic [32P]phosphate substitute as the phosphate donor, demonstrating that transfer of phosphate from ATP to protein is direct and extracellular. The macrophage-associated protein kinase was also shown to phosphorylate added acceptor protein (histone) and to be tightly associated with the cell surface. Thus, a new ecto-enzyme, a protein kinase, has been detected in macrophages.
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