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Fc-Receptor Mediated Protein Phosphorylation in Murine Peritoneal Macrophages*

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The effect of Fc receptor engagement on protein phosphorylation in murine peritoneal macrophages has been investigated. Treatment of macrophage cultures with insoluble immune complexes resulted in enhanced phosphorylation of six proteins at 73, 66, 53, 37, 31 and 25 kD. Comparison of the protein phosphorylation patterns induced by immune complexes with those induced by agents which mimic the actions of well known intracellular second messengers (i.e., A23187, dibutyryl cAMP, or phorbol myristate acetate) revealed substantial similarity between Fc receptor induced events and those induced in response to phorbol diesters. There were, however, two phosphorylated proteins which were only seen following stimulation with immune complexes. Thus, more than one kind of protein kinase activity appears to be involved in Fc receptor mediated stimulation of macrophage function.

The engagement of macrophage cell surface receptors capable of interacting with the Fc portion of immunoglobulins (Ig) promotes phagocytosis of Ig coated particles (1), stimulates the secretion of a wide variety of products including reactive oxygen intermediates, metabolites of arachidonic acid, and lysosomal hydrolases (2,3), and may result in antibody dependent, cell mediated cytolysis of tumor cells (4). The molecular mechanisms by which Fc receptors (FcR) mediate transmission of surface stimulatory signals to the macrophage interior, and the subsequent events which ultimately lead to functional output are poorly defined. Signal transduction involves the interaction of ligands with specific receptors which are coupled to various effector or amplifier systems responsible for generating internal signals or secondary messengers (5). The reversible covalent modification of proteins by protein kinases or phosphatases (i.e., phosphorylation, dephosphorylation) constitutes a major regulatory process for control of intracellular events by external stimuli (6). In the present studies, we examined the

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phosphorylation of macrophage proteins in response to the engagement of FcR using insoluble immune complexes and compared it to that stimulated by pharmacologic agents used to mimic known second messengers.

Materials and Methods

Preparation and Culture of Peritoneal Macrophages

C57Bl/6J mice (6-8 weeks of age) were obtained from the Trudeau Institute (Saranac Lake, NY) and were used as the source of macrophages in all experiments. Macrophages were isolated from mice injected with proteose peptone broth, thioglycollate broth, or methyl vinyl ether copolymer-2 (MVE-2), or from mice chronically infected with bacillus Calmette Guerin (BCG, Phipps strain 1029, Trudeau Institute) using methodology previously described (7). Peritoneal cells were plated at 3 x 10^6/4.5 cm^2 plastic tissue culture wells in RPMI 1640 medium supplemented with 10% fetal bovine serum, glutamine, penicillin, and streptomycin.

Preparation of Immune Complexes

Insoluble immune complexes were prepared by incubating 100 µl of 1 x 10^6M human transferrin (Sigma Chemical Co., St. Louis, MO) with 900 µl of rabbit anti human transferrin IgG (5 mg/ml) (Cappell Laboratories, Cochranville, PA) for 1-2 hrs at 4°C. The resulting precipitates were washed twice by centrifugation and resuspension in distilled water, and added to macrophage cultures in the indicated amounts.

Phosphorylation of Macrophage Proteins

Adherent macrophage cultures were washed 4x with Tyrode's buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 1 mM $MgCl_2$, 0.05 mM NaH_2PO_4, 5 mM glucose, 5 mM HEPES, pH 7.4). Then 250 µCi of ^{32}P-orthophosphoric acid (New England Nuclear, Boston, MA) in 200 µl Tyrode's buffer were added to each culture simultaneously with the relevant stimulus. Such cultures were incubated at 37°C for 30 min, washed 5x with ice cold Tyrode's buffer and solubilized in electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8, 4% Na dodecyl sulfate, 20% glycerol, and 10% 2-mercaptoethanol), boiled for 2 min and analyzed on 10% polyacrylamide gels according to the method of Laemmli (8). Coomassie blue stained gels were subsequently dried under vacuum and exposed for appropriate times using Kodak X-omat RP-5 X-Ray film with Dupont "lightening plus" intensifying screens. Stimuli of phosphorylation were prepared as follows: phorbol-12,13-myristate acetate (PMA) and calcium ionophore (A23187) were prepared as stock solutions of 1 mg/ml in DMSO and were diluted in Tyrode's buffer immediately prior to use. The final concentration of DMSO in the experiment was 0.1% which had no effect upon macrophage protein phosphorylation. N^6,O^2-dibutyryl-adenosine-3',5'-cyclic monophosphate was prepared as a stock solution of 10 mM in Tyrode's buffer. Monensin was prepared as a stock solution of 10 mM in ethanol and was diluted in Tyrode's buffer immediately prior to use. The final concentration of ethanol was 0.1% which had no effect upon macrophage protein phosphorylation. Prostaglandin E_2 was prepared as a stock solution 5 mM in Tyrode's buffer.

Results and Discussion

On MVE-2 elicited macrophages, engagement of FcR using insoluble immune complexes results in an overall increase in phosphorylation of proteins when compared to unstimulated macrophages (Fig. 1, Lanes 1 and 2). New or enhanced phosphoprotein bands can be seen at 73,66,53,37,31 and 25 kD. Similar or identical patterns of FcR

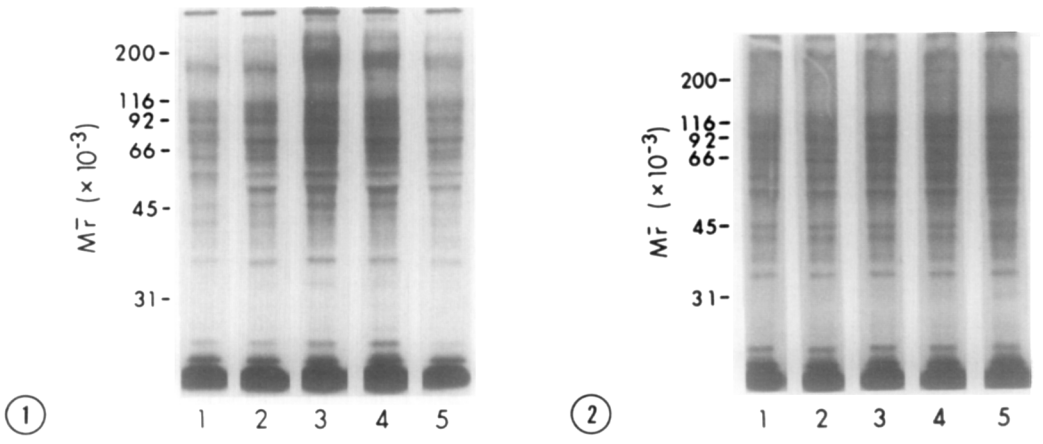

Figure 1 Autoradiograph of macrophage phosphoproteins ^{32}P-labeled and electrophoretically separated on 10% acrylamide gels as described in the methods. Lane 1: unstimulated MVE-2 elicited macrophages. Lanes 2 through 5: stimulation with 40 µg of insoluble immune complexes of (lane 2) MVE-2 elicited macrophages, (lane 3) proteose peptone elicited macrophages, (lane 4) thioglycollate broth elicited macrophages, and (lane 5) BCG activated macrophages.

Figure 2 Autoradiograph of MVE-2 elicited macrophage (^{32}P) phosphoproteins stimulated for 30 min by the addition of the following amounts of insoluble immune complexes: Lane 1: unstimulated, Lane 2: 0.2 µg, Lane 3: 2 µg, Lane 4: 20 µg and Lane 5: 40 µg.

stimulated protein phosphorylation were also observed when proteose peptone elicited, thioglycollate broth elicited or BCG activated macrophages were used (Fig. 1, lanes 3-5).

The specific changes in phosphorylation observed in response to immune complexes are dependent upon the amount of stimulus added (Fig. 2). Lanes 1-5 show the phosphorylation observed in response to 0, 0.2, 2, 20, and 40 µg of added insoluble immune complexes. Enhanced phosphorylation is seen to be stimulated by as little as 2 µg and is optimal in the 20 to 40 µg range. The phosphorylation response also shows a time dependence (Fig. 3). Lane 1 shows the phosphorylation response in control macrophage cultures which received no stimulus. Lanes 2-5 show the phosphorylation response in macrophage cultures which received 40 µg of immune complexes for 5, 10, 15, and 30 min respectively. Enhanced phosphorylation is discernible at 5 min, is

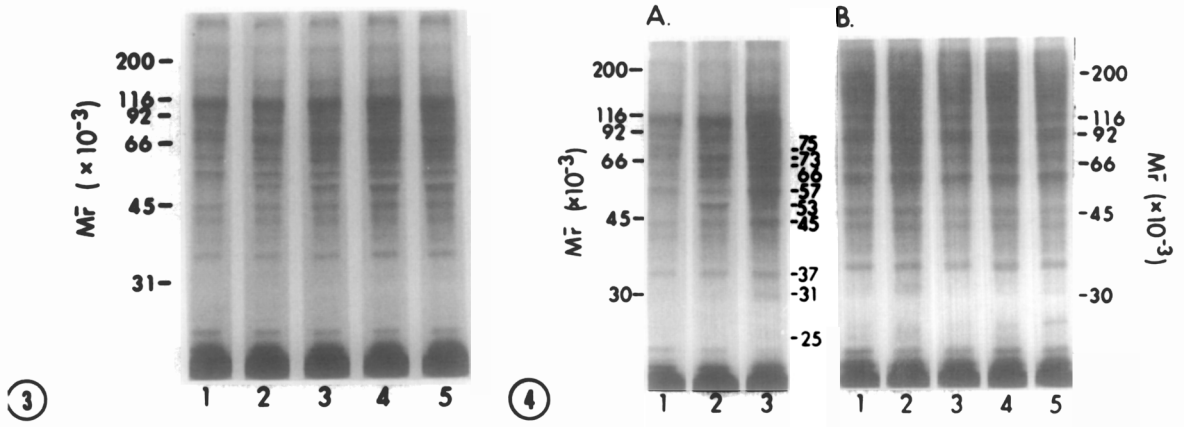

Figure 3 Autoradiograph of MVE-2 elicited macrophage (^{32}P) phosphoproteins stimulated by the addition of 40 µg of insoluble immune complexes for the following time periods: Lane 1: unstimulated, Lane 2: 5 min, Lane 3: 10 min, Lane 4: 15 min, Lane 5: 30 min.

Figure 4 a & b Autoradiograph of MVE-2 elicited macrophage (^{32}P) phosphoproteins stimulated for 30 min by the addition of the following agents: Fig. 4a, Lane 1: unstimulated, Lane 2: 40 µg of insoluble immune complexes, and Lane 3: 100 ng/ml PMA; Fig. 4b, Lane 1: unstimulated, Lane 2: 0.1 mM $CaCl_2$ plus 1 µM A23187, Lane 3: 1 µM PGE_2, Lane 4: 1 µM Monensin and Lane 5: 1 mM dibutyryl cyclic AMP.

maximal by 15 min and is maintained through 30 min. All cultures were exposed to $^{32}PO_4$ for the same time period (i.e., 30 min).

Protein phosphorylation may be regulated by intracellular second messengers which are generated following the interaction of exogenous signals with specific cell surface receptors. The most thoroughly evaluated include altered levels of cyclic AMP, changes in intracellular Ca^{++} concentrations, and more recently the breakdown products of phosphatidylinositol-4,5-bisphosphate (i.e., 1,4,5-inositoltriphosphate, and diacylglycerol). In order to identify which second messenger(s) might be responsible for the FcR mediated protein phosphorylation events seen in Figures 1-3, we tested the effect of pharmacologic agents which mimic the action of these known second messengers. MVE-2 elicited macrophages were either untreated or were treated with immune complexes or PMA (Fig. 4A) or with A23187 plus Ca^{++}, dibutyryl cyclic AMP, monensin (a monovalent cation ionophore), or prostaglandin E_2 (Fig. 4B). While some enhanced phosphorylation was observed with A23187 and with dibutyryl cyclic AMP (Fig.

Table 1 Summary of Macrophage Protein Phosphorylation Induced By Immune Complexes or PMA

	Stimulus	
MF [x 10^{-3}]	PMA	Immune Complexes
75	+	-
73	-	+
66	+	+
57	+	-
53	-	+
45	+	-
37	+	+
31	+	+
25	+	+

4B, lanes 2,5), only PMA induced changes comparable to those induced by immune complexes (Fig. 4A, lanes 2 and 3, table 1). Phosphoproteins at 25, 31, 37, and 66 kD appear in response to either stimulus. Phosphorylation of proteins of 45, 57, and 75 kD are induced only by PMA while those at 53 and 73 kD are stimulated only with immune complexes.

While others have reported that FcR engagement leads to elevation in cyclic AMP concentrations either directly or via secretion of prostaglandin E_2 (9), or functions itself as a monovalent cation gate (10), our findings suggest that Ca^{++} dependent or cyclic AMP dependent protein kinases are not involved in FcR mediated protein phosphorylation. However, PMA mediated stimulation of protein phosphorylation was remarkably similar to that induced by immune complexes. In light of recent evidence that PMA (and other active phorbol diesters) act by binding to and activating a Ca^{++}, phospholipid dependent protein kinase (protein kinase C) (11,12), these results suggest that FcR may be coupled either directly or indirectly to this particular protein kinase. Because protein kinase C is an important effector molecule in the phosphatidyl inositol signal transduction pathway (13), FcR engagement might normally trigger this cycle. However the major proteins phosphorylated only in response to stimulation with immune

complexes (53 and 73 kD) are not induced by any of the pharmacologic stimuli tested suggesting that protein kinase(s) other than those discussed above may be involved.

Although no direct links can as yet be drawn between the stimulation of protein phosphorylation and stimulation of function by FcR engagement, it is noteworthy that both the dose response and the time dependency for immune complexes are consistent with their involvement in H_2O_2 secretion and arachidonic acid metabolism (2,3). The possible role of protein phosphorylation (FcR mediated and otherwise) in the control of macrophage functional output suggested by the observations reported above will require further study.

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