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Identification and subcellular localization of proteins that are rapidly phosphorylated in tyrosine in response to colony-stimulating factor 1

(receptor/growth factor/protein tyrosine kinase/macrophage)

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ABSTRACT **To investigate growth factor-mediated signal transduction, we have studied phosphorylation events that take place within seconds of the binding of colony-stimulating factor 1 (CSF-1) to its cell-surface receptor. CSF-1 stimulated rapid tyrosine phosphorylation of cellular proteins in murine BAC1.2F5 macrophages at 37°C and 4°C. The pattern of CSF-1-stimulated tyrosine phosphorylation of at least 15 different proteins at both temperatures was similar and unchanged by treatment of the lysate with reducing agent. With the exception of the 185-kDa CSF-1 receptor, a 260-kDa protein and a 133-kDa protein, the proteins were predominantly cytoplasmic. At 37°C, all the proteins were phosphorylated within 30 sec of addition of growth factor. At 4°C, CSF-1 receptor sites were saturated after 2 min of incubation in the presence of high concentrations of CSF-1 and differences in the order of appearance of phosphorylated proteins were observed: 185 kDa (CSF-1 receptor) (by 2 min); 99 kDa (by 4 min); 125 kDa (by 10 min); 61 kDa (by 30 min); and 260 kDa, 84 kDa, and 41 kDa (by 180 min). In addition to stimulating the phosphorylation of these proteins in tyrosine, CSF-1 caused dephosphorylation of phosphorylated serine residues on the receptor. As neither CSF-1 nor its receptor is internalized at 4°C, analysis of these early reactions and the phosphotyrosine-containing proteins in intact cells under these conditions should lead to an understanding of the early events in growth factor receptor-mediated signal transduction.**

The purification and cloning of the receptor for several growth factors, including epidermal growth factor, insulin, platelet-derived growth factor, colony-stimulating factor 1 (CSF-1), and insulin-like growth factor have indicated that they are tyrosine kinases, possessing an extracellular ligand-binding domain and an intracellular tyrosine kinase domain (reviewed in ref. 1). Furthermore, recent studies with the receptors for insulin and epidermal growth factor indicate that their tyrosine kinase activity is essential for signal transduction (2–4). Thus, a major question concerning the action of these growth factors is the nature and function of the intracellular physiological substrates of their receptor kinases.

CSF-1 selectively regulates the survival, proliferation, and differentiation of mononuclear phagocytic cells (reviewed in ref. 4). It may also, via its action on trophoblastic cells, regulate the formation and maintenance of the placenta (5, 6). The CSF-1 receptor is a tyrosine kinase (7), which has been shown to be the c-*fms* protooncogene product (8–10). The mechanism by which CSF-1 stimulates survival and proliferation can be conveniently studied in the CSF-1-dependent murine macrophage cell line BAC1.2F5 (11, 12). In this paper, we describe conditions for CSF-1 stimulation at 4°C, under which the very early postreceptor tyrosine phosphorylation events in the CSF-1 response in BAC1.2F5 cells can be studied. This temperature not only slows down the rate of these phosphorylation reactions but also prevents ligand or receptor internalization (13). We also identify several proteins that are very rapidly phosphorylated in tyrosine in response to CSF-1 and that may play an important role in the regulation of cells by this growth factor.

MATERIALS AND METHODS

Cell Culture and Labeling. BAC1.2F5 cells were cultured as described (11). Confluent cultures (6×10^6 cells per 10-cm-diameter tissue culture dish) were cultured in medium without CSF-1 for 18 hr (receptor up-regulation) and then incubated for 2 hr in phosphate-free modified minimal essential medium with Earle's salts supplemented with nonessential amino acids and containing 25 mM Hepes buffer (Irvine Scientific), L-glutamine (292 μg/ml), 50 μM 2-mercaptoethanol, penicillin (0.2 g/liter), streptomycin (0.2 g/liter), and 10% heat-inactivated dialyzed (against 150 mM NaCl, four changes, each of 20 vol, for 48 hr at 4°C) fetal calf serum after washing three times in the same medium. Cells were labeled by incubation for 2 hr in the above medium containing 1/10 the amount of $NaHCO_3$ (to reduce pH change during long-term incubations at 4°C) and carrier-free *o*-[^{32}P]phosphate (1 mCi/ml, 9000 Ci/mmol, 3 ml per dish; 1 Ci = 37 GBq).

CSF-1 Stimulation. After equilibrating the ^{32}P-labeled cells at the desired temperature, purified L-cell CSF-1 (14) was added in 300 μl of the labeling medium (30,000 units/ml; final concentration, 13.2 nM). For analysis of protein phosphorylation in whole cells, the reaction was stopped by rapid aspiration and washing (seven times, ≤1 min total) with ice-cold phosphate-buffered saline (0.14 mM NaCl/3 mM KCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.4) (PBS) followed by solubilization of the monolayer by addition of 2 ml of ice-cold extraction buffer [10 mM Tris/50 mM NaCl/1% Triton X-100/30 mM sodium pyrophosphate/50 mM NaF/5 mM EDTA/100 μM sodium orthovanadate/1 mM phenylmethylsulfonyl fluoride/0.1% bovine serum albumin (crystallized and lyophilized; Sigma), pH 7.6]. Cell debris was removed by centrifugation (11,000 × *g*, 30 min, 4°C). Subcellular fractionation was performed as described (15) except that all buffers contained 10 mM NaF, 10 mM sodium pyrophosphate, and 500 μM sodium orthovanadate, and cells

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Abbreviation: CSF-1, colony-stimulating factor 1.

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were released from the dishes by scraping in ice-cold PBS containing 4 mM iodoacetic acid and 1 mM EGTA (as -SH and Ca^{2+}-activated protease inhibitors) prior to collection by centrifugation (400 × *g*, 5 min, 4°C).

Isolation and Characterization of Phosphotyrosine-Containing Proteins. Cell extracts (1 ml) were incubated with 25 μl of packed Sepharose 4B to which monoclonal 1G2 anti-phosphotyrosine antibody (anti-phosphotyrosine) (ref. 16; A.R.F., Jr., M. R. Posner, & F. Mermelstein, unpublished data) had been covalently coupled. Phosphotyrosine-containing proteins were competitively eluted in 40 μl of 1 mM phenylphosphate-containing buffer at 4°C as described (16). The phosphotyrosine-containing proteins in the eluate were separated by $NaDodSO_4$/PAGE according to the method of Laemmli (17). Analysis of phosphorylated amino acids was carried out by partial acid hydrolysis and high-voltage electrophoresis as described by Golden *et al.* (18), with the following modifications. Gel bands were excised, washed with 10% methanol (three times, 10 min each), dried in a Speed-Vac, and hydrolyzed (110°C, 2 hr) in 6 M HCl with internal phosphotyrosine, phosphoserine, and phosphothreonine standards (50 μg per sample). After electrophoretic separation of the phosphorylated amino acids, the paper was stained with 0.5% (wt/vol) ninhydrin in a 30%:70% (vol/vol) CH_3COOH/acetone mixture. The individual spots were excised and extracted with 2 ml of Methyl Cellosolve (Pierce Chemicals) and the extracts were read at 575 nm in a spectrophotometer. The percentage destruction of each phosphorylated amino acid was calculated by comparison with eluates from electrophoreses of equivalent amounts of nonhydrolyzed phosphorylated amino acids. Staphylococcal V8 protease (type XVIII, Sigma) phosphopeptide mapping was carried out as described by Cleveland *et al.* (19).

^{125}I-Labeled CSF-1 Binding. ^{125}I-labeled CSF-1 (≈300,000 cpm per ng of protein) was prepared as described (14) and used to trace label purified murine CSF-1 (14). Binding of ^{125}I-labeled CSF-1 was carried out at 4°C as described (14) in 35-mm culture dishes containing 10^6 confluent cells that had been incubated as described above for the preparation of ^{32}P-labeled cells, except that the *o*-[^{32}P]phosphate was omitted. The final concentration of CSF-1 was 3.2 nM.

RESULTS

CSF-1-Stimulated Protein Phosphorylation at 37°C. CSF-1-starved cells were preincubated with $^{32}P_i$ for 2 hr and incubated with 13.2 nM CSF-1 for 30 sec, 2 min, 4 min, or 30 min at 37°C. Phosphotyrosine-containing proteins were isolated by anti-phosphotyrosine antibody affinity chromatography from cell lysates and analyzed by $NaDodSO_4$/PAGE. As shown in Fig. 1, CSF-1 either induced or increased the transient appearance of several anti-phosphotyrosine precipitable phosphorylated proteins (including proteins of 260, 185, 155, 125, 111, 99, 84, 70, 64, 61, 57, 54, 41, 36, 34, and 21 kDa). Increased phosphorylation of all these proteins was reproducible and apparent within 30 sec of CSF-1 addition. Some proteins (e.g., 185, 99, 61, 54, and 41 kDa) appeared to be maximally phosphorylated by 30 sec, some (260, 125, 36, and 34 kDa) by 2 min, and others (111 kDa) by 4 min after CSF-1 addition. By 30 min at 37°C, the CSF-1 receptor is maximally down-regulated (13) and the CSF-1 effect on the phosphorylation of the receptor [185 kDa, identified by immunoprecipitation with anti-v-fms antiserum (ref. 8; Table 1; data not shown)] and some proteins was barely apparent, whereas the CSF-1-increased phosphorylation of others was still intense.

CSF-1-Stimulated Phosphorylation at 4°C. Because of the rapid, transient, and differential nature of the CSF-1-induced tyrosine phosphorylation of proteins observed at 37°C and because we were interested in studying the very early effects of CSF-1, it was of interest to determine whether the reaction rates could be slowed down by lowering the temperature to 4°C. As shown in Fig. 1 (lanes 9 and 10), phosphorylation of most of the proteins was observed at 4°C after a 30-min incubation at the same concentration of CSF-1. Furthermore, exactly the same pattern of banding was observed when the sample was reduced (0.1 M 2-mercaptoethanol, 5 min, 100°C in $NaDodSO_4$/PAGE sample buffer) prior to electrophoresis (lanes 11 and 12), indicating that none of the phosphorylated proteins was covalently attached to any protein by disulfide bonds.

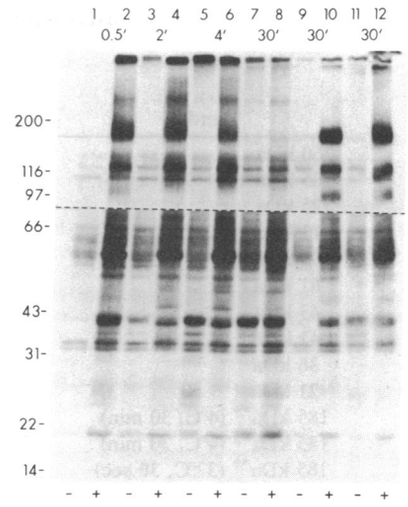

FIG. 1. Kinetics of CSF-1-stimulated phosphorylation of anti-phosphotyrosine-reactive proteins at 37°C and comparison with the pattern of their phosphorylation at 4°C. Autoradiograph of gradient (5–15% acrylamide) $NaDodSO_4$/PAGE of anti-phosphotyrosine-reactive proteins from lysates of cells preincubated with $^{32}P_i$ for 2 hr at 37°C and incubated with (+) or without (−) CSF-1 for the indicated times at 37°C (lanes 1–8) or 4°C (lanes 9–12). Samples in lanes 11 and 12 were not subjected to the usual reduction procedure (100°C, 5 min, 0.1 M 2-mercaptoethanol). Exposure times: above dashed line, 8 hr; below dashed line, 52 hr. The increased intensity of bands from control cells during the time course is possibly due to slight differences in the time at 4°C between washing and lysis for pairs of treated and untreated dishes. Numbers on left are kDa.

Kinetics of CSF-1-Stimulated Phosphorylation at 4°C. We wished to investigate the kinetics of the appearance and disappearance of phosphorylated proteins in relation to receptor phosphorylation induced by ligand binding. We therefore exposed cells to a concentration of CSF-1 (13.2 nM) that resulted in rapid saturation of the receptors at 4°C. Under these conditions, 75% of the receptors were saturated within 60 sec and 100% were saturated within 120 sec of CSF-1 addition (Fig. 2). At 13.2 nM CSF-1, there was no major difference between cells lysed and processed with (Fig. 3*a*) or without (Fig. 3*b*) 100 μM sodium orthovanadate. However, kinetic differences in the CSF-1-induced phosphorylation of bands were apparent. The order of appearance of the major phosphorylated proteins was 185 kDa (CSF-1 receptor) (2 min); 99 kDa (4 min); 125 kDa (10 min); 61 kDa (30 min); and 260, 84, and 41 kDa (180 min). Scanning of an appropriate autoradiograph from the experiment in Fig. 3*b* revealed that 50% of the area under the 185-, 99-, and 125-kDa peaks at 180 min (maximal stimulation) was achieved at ≈4 min, ≈10 min, and ≈30 min of stimulation, respectively.

Characterization of the Phosphorylated Proteins. To confirm that all of the major phosphoproteins were phosphorylated at tyrosine residues, each of the excised bands from lanes containing CSF-1-stimulated (4°C) cell lysate together with corresponding regions from lanes containing unstimulated lysate was subjected to analysis of phosphorylated

Table 1. Analyses of excised bands

Band	Phosphotyrosine, cpm		Phosphothreonine, cpm		Phosphoserine, cpm	
	−CSF-1	+CSF-1	−CSF-1	+CSF-1	−CSF-1	+CSF-1
260 kDa	12	42	2	15	12	36
185 kDa	10	144	6	19	18	222
125 kDa	11	102	4	16	39	121
111 kDa	2	13	6	5	38	56
99 kDa	7	62	0	7	13	29
61 kDa	0	49	0	1	0	4
43 kDa	1	17	0	9	14	30
41 kDa	0	53	0	2	6	16
36 kDa	7	34	1	8	8	26
21 kDa	0	17	2	0	0	0
185 kDa*† (4°C, 30 min)	0	37	2	4	234	190
185 kDa*‡ (4°C, 30 min)	0	39	0	2	0	61
185 kDa*† (37°C, 30 sec)	0	32	0	9	218	190

Average of determinations on duplicate bands, corrected for the destruction of phosphotyrosine (70%), phosphothreonine (5%), and phosphoserine (20%) during hydrolysis. Duplicates higher than 17 cpm did not differ by >10%.

*Results of a separate experiment in which the 185-kDa receptor band was obtained from NaDodSO$_4$/PAGE of anti-v-fms antiserum immunoprecipitates (†) (8) or anti-phosphotyrosine-reactive proteins (‡). The 185-kDa species and the phosphotyrosine-containing 185-kDa species, respectively, were quantitatively recovered. The temperature and time of CSF-1 stimulation are in parentheses.

amino acids (Table 1). Each of the major CSF-1-stimulated phosphoprotein bands had counts in phosphotyrosine and each had markedly greater counts in phosphotyrosine than corresponding regions from the lanes containing unstimulated lysate. Also shown in Table 1 are analyses of phosphorylated amino acids of the CSF-1 receptor band from gels of immunoprecipitated receptor. At either 4°C or 37°C, CSF-1 not only stimulated incorporation into tyrosine residues but consistently decreased incorporation into serine residues of the receptor. Analyses of total cell lysates indicated that CSF-1 stimulated a 1.5- to 2-fold increase in phosphotyrosine counts at 4°C (5–6% of the total ^{32}P incorporated into protein in the presence of CSF-1) (data not shown).

To assess whether the major phosphotyrosine proteins were related to each other, they were subjected to V8 protease phosphopeptide mapping (Fig. 4). Each of the phosphoprotein bands examined possessed unique phosphopeptide banding patterns. (The phosphopeptide band at 66 kDa in digests of the 185-, 125-, 99-, and 61-kDa phosphoprotein bands is an artifact caused by a carrier protein, bovine serum albumin, which appears to have resisted degradation.)

As the molecular masses of several of the phosphotyrosine-containing proteins corresponded to the sizes of proteins encoded by known tyrosine kinase protooncogenes, the ability of antisera that react with either c-*src*- (20), c-*abl*- (21), or c-*fes/fps* (22)-encoded proteins to immunoprecipitate any of the CSF-1-stimulated phosphotyrosine-containing proteins was tested. Under conditions in which proteins of the appropriate size could be precipitated from total lysate and the flow-through fractions of the anti-phosphotyrosine antibody column, no phosphorylated proteins could be precipitated from the phosphotyrosine-containing protein fraction (data not shown). However, CSF-1 increased serine phosphorylation of the 60-kDa band precipitated by the anti-src antiserum even though phosphotyrosine could not be detected under the conditions used (data not shown).

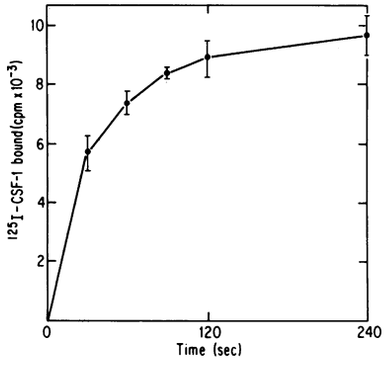

FIG. 2. Kinetics of ^{125}I-labeled CSF-1 binding to cells under the conditions used for CSF-1-stimulated phosphorylation in intact cells at 4°C. CSF-1 concentration, 13.2 nM. Means ± SD; three dishes per point.

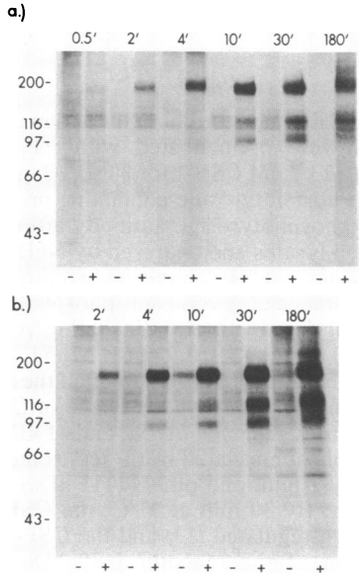

FIG. 3. Kinetics of CSF-1-stimulated phosphorylation of anti-phosphotyrosine-reactive proteins at 4°C. Autoradiograph of NaDodSO$_4$/PAGE (7.5% acrylamide) of anti-phosphotyrosine-reactive proteins from lysates of cells incubated with (+) or without (−) 13.2 nM CSF-1 for the indicated times. Lysis and isolation in the presence (*a*) or absence (*b*) of 100 μM sodium orthovanadate (separate experiments). (*a*) Composite of autoradiographs from two separate gels. Exposure time, 20 hr. Numbers on left are kDa.

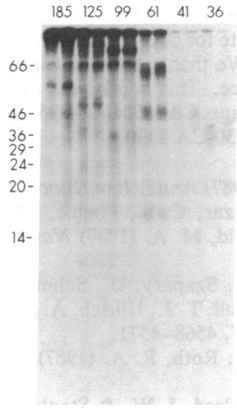

FIG. 4. V8 protease phosphopeptide maps of several CSF-1-stimulated phosphotyrosine-containing proteins. Autoradiograph of $NaDodSO_4$/PAGE (15% acrylamide) of V8 protease-digested excised phosphoprotein bands. Numbers above lanes are sizes of excised bands (kDa). Numbers on left are kDa.

Cellular Localization of the Phosphotyrosine-Containing Proteins. To determine the cellular localization of the phosphotyrosine-containing proteins, membrane, cytoplasmic, and nuclear fractions of cells stimulated by CSF-1 at 4°C were analyzed. Fig. 5 shows that the phosphotyrosine-containing proteins were predominantly cytoplasmic. As expected, at this temperature no CSF-1-stimulated phosphotyrosine-containing proteins were detected in the nuclear fraction (Fig. 5*b*) other than those apparently derived from cellular contamination. (The phosphoprotein band at 43 kDa is an artifact caused by a carrier protein, ovalbumin.) Only four phosphotyrosine-containing proteins were represented in proportionately significant amounts in the membrane fraction (Fig. 5*a*; data not shown). These were the 185-kDa CSF-1 receptor, which, as expected, was exclusively represented in this fraction and the 260-, 145-, and 133-kDa proteins. Minor proportions of the 99-, 84-, 64-, 61-, 43-, and 36-kDa bands were also recovered in the membrane fraction. To improve

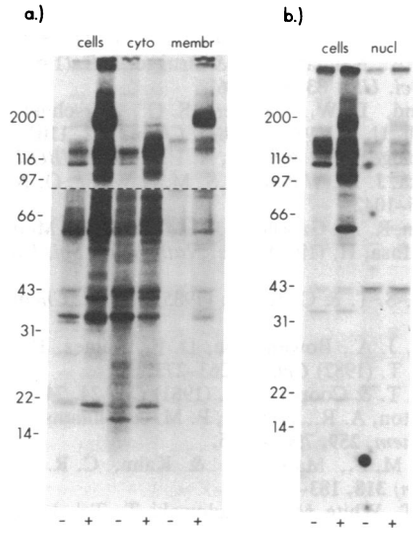

FIG. 5. Autoradiograph of gradient (5–15% acrylamide) $NaDodSO_4$/PAGE of the anti-phosphotyrosine-reactive proteins of subcellular fractions of cells stimulated (30 min, 4°C) with (+) and without (−) CSF-1. (*a*) Intact cells (from 2×10^6 cells) and cytoplasmic (cyto, from 3×10^6 cells) and membrane (membr, from 3×10^6 cells) fractions of one homogenate from them (exposure times: above dashed line, 17 hr; below dashed line, 54 hr). (*b*) Intact cells and nuclear (nucl) fraction (another experiment; exposure time, 20 hr). Numbers on left are kDa.

our ability to discriminate between the different phosphotyrosine-containing proteins, the whole cell lysate, cytoplasmic, and membrane fractions were subjected to two-dimensional gel electrophoresis (data not shown). After stimulation, spots visible in the absence of CSF-1 were found to be more acidic, consistent with their phosphorylation at several new sites. However, analysis of these gels did not reveal the presence of species not resolved by subcellular fractionation and one-dimensional analysis.

DISCUSSION

We have studied protein tyrosine phosphorylation in whole cells in response to rapid saturation of their CSF-1 receptors with CSF-1. CSF-1 stimulates rapid tyrosine phosphorylation at 37°C. While it is possible to demonstrate differential phosphorylation of some protein bands within the first 4 min at 37°C, all bands were strongly phosphorylated within 30 sec of stimulation. By using a high ligand concentration and stimulation at 4°C, the kinetics of binding of CSF-1 to the receptor was clearly shown to precede the CSF-1-stimulated phosphorylation of the receptor. Maximal binding was achieved at 2 min. This was the earliest time point at which receptor phosphorylation in tyrosine was detected and 10–30 min before maximal phosphorylation of the receptor was achieved. The conditions also enabled us to demonstrate significant differences in the order of phosphorylation of the major (185, 99, and 125 kDa) phosphotyrosine-containing species.

The effect of lowering the temperature to 4°C appears to be primarily one of lowering the rates of phosphorylation and dephosphorylation observed at 37°C. As most of the bands phosphorylated at 37°C were phosphorylated at 4°C and analyses of phosphorylated amino acids of the CSF-1 receptor immunoprecipitated with anti-v-fms antiserum were very similar, it would appear that the initial events at 4°C are equivalent to those occurring at 37°C. This is a significant observation as these initial phosphorylation reactions are more easily studied at 4°C. An additional advantage is that at 4°C it is possible to study signal transduction in the absence of internalization of receptor or growth factor (13).

Analysis of phosphorylated amino acids of the anti-v-fms immunoprecipitated CSF-1 receptor band revealed that, in addition to the expected increase in phosphotyrosine, there was a consistent decrease in phosphoserine after stimulation with CSF-1. This suggests that CSF-1 activates a serine phosphatase, although the result might be explained by a depletion of the cellular ATP pool due to the activated receptor tyrosine kinase. If receptor phosphorylation on serine residues had been independent of tyrosine phosphorylation, the data in Table 1 would allow us to estimate the fraction of CSF-1 receptor phosphorylated in tyrosine—i.e., the phosphoserine counts in the phosphotyrosine-containing receptor fraction divided by the phosphoserine counts in the anti-v-fms immunoprecipitated (total) receptor fraction, or 61/190 (32%). Because of the associated CSF-1-induced serine dephosphorylation of the receptor, this figure must be a slight underestimate. Nevertheless, it agrees closely with the published estimate of the fraction of CSF-1 receptors expressed at the up-regulated macrophage cell surface at 4°C (≈33%; ref. 13).

V-8 protease phosphopeptide mapping of several of the protein bands indicated that they bear no precursor/product relationships and are unrelated proteins. Preliminary two-dimensional gel analysis indicated that most, if not all, of these proteins were resolved in one-dimensional gradient $NaDodSO_4$/PAGE, although the behavior of some species was consistent with their phosphorylation at multiple sites, as also indicated by the detection of phosphoserine as well as phosphotyrosine in their analyses. While they failed to be

recognized by antibodies to several known tyrosine kinases it will be important to determine whether they are precipitated by antisera to other known proteins. Interestingly, by comparison with the CSF-1 receptors, the majority of these proteins appear to be cytoplasmic, although the conditions used for subcellular fractionation do not preclude their association with the membrane in intact cells.

Although the identity of non-CSF-1 receptor phosphotyrosine-containing proteins is unknown, the protein at 111 kDa appears to be similar to a phosphotyrosine-containing protein described as 106 kDa in chronic myelogenous leukemia cells but contained in virtually all mammalian cells (ref. 16; A.R.F., Jr., unpublished data). As in other cells, this protein has a high ratio of phosphoserine to phosphotyrosine and an acidic isoelectric point of ≈5.5 (Table 1; data not shown). Proteins at 36, 41, and 43 kDa may be related to proteins of similar sizes that are tyrosine phosphorylated in response to a number of other mitogenic stimuli (23, 24).

Similar studies of growth factor-induced tyrosine phosphorylation have been carried out with epidermal growth factor (25), platelet-derived growth factor (26), insulin (27) and insulin-like growth factor (28). At 37°C, within 5 min of addition of the growth factor, they all showed rapid tyrosine phosphorylation of the receptor and some other cellular proteins. A preliminary report of CSF-1-induced protein phosphorylation in BAC1.2F5 cells described the phosphorylation of proteins of 110, 100, 66, 53, 45, and 36 kDa detected by immunoblotting with anti-phosphotyrosine antibodies (29). In the present study, additional bands have been clearly and reproducibly identified, consistent with the failure of the direct immunoblotting technique to detect several of the phosphotyrosine-containing proteins, including the CSF-1 receptor (29). In a related study, Morrison *et al.* (30) reported on the *in vivo* phosphorylation associated with transformation by the v-*fms* oncogene. In v-*fms*-transformed NIH 3T3 cells, increased phosphorylation of 200-, 185-, 140- (gp140 v-*fms*), 110-, 115-, 92-, 75-, 85-, 50-, 41-, and 30-kDa proteins could be detected by immunoblotting with anti-phosphotyrosine antibody. Some of these proteins were of similar size to proteins whose phosphorylation was increased by platelet-derived growth factor (30) and by CSF-1 (ref. 29; this paper), indicating that some of the substrates involved in these systems are potentially common.

As has been observed in the platelet-derived growth factor receptor system (26), lowering the temperature to 4°C slows the rate of tyrosine phosphorylation. In the CSF-1-receptor system, phosphorylation comparable in intensity to phosphorylation at 37°C can be achieved by incubation for a longer time period (the degree of phosphorylation after incubation for 180 min at 4°C approximates that found after a 2-min incubation at 37°C) and differences in the order of tyrosine phosphorylation of individual proteins can be resolved. Studies at 4°C in this system therefore provide a means of studying the kinetics of phosphorylation events stimulated by CSF-1. Furthermore, as phosphorylation of the 99-kDa band is reproducibly much more intense at 4°C than at 37°C, it would appear that some bands that are transiently phosphorylated at 37°C will be more easily identified at 4°C, possibly because of differential inhibition of their dephosphorylation at the lower temperature. Finally, these experiments at 4°C indicate that a large number of phosphorylation events take place in the absence of any receptor or ligand internalization. As these events are very early in the growth factor response, their analysis warrants further investigation.

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