Melanoma Growth Stimulatory Activity Signaling through the Class II Interleukin-8 Receptor Enhances the Tyrosine Phosphorylation of Crk-Associated Substrate, p130, and a 70-Kilodalton Protein†

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ABSTRACT: Binding of the CXC chemokine, melanoma growth stimulatory activity (MGSA), to the class II IL-8 receptor on cells which overexpress this G-protein coupled receptor results in enhanced phosphorylation on serine residues. In experiments described herein, it is demonstrated that MGSA also enhances the tyrosine phosphorylation of two endogenously tyrosine phosphorylated proteins approximately 130 and 70 kDa in size. MGSA treatment (5 nM) of the clonally selected, stably transfected placental cell line, 3ASubE P-3, which overexpresses the class II IL-8 receptor, results in the maximal tyrosine phosphorylation of the 130 kDa protein before 2 min. This enhanced phosphorylation of the 130 kDa protein returns to basal level after a 5 min treatment. Based upon cell fractionation studies, the 130 kDa protein is concentrated in the membrane fraction of the cells. The 70 kDa protein which also shows tyrosine phosphorylation is predominately cytosolic. The identity of the 130 kDa tyrosine phosphorylated protein was determined by immunoprecipitation and Western blot analyses. In these experiments, the 130 kDa tyrosine phosphorylated protein was shown to immunoprecipitate with antibody to the cas antigen (crk-associated substrate) and with antibody to the the p130 tyrosine phosphorylated protein described as undergoing tyrosine phosphorylation in src transformed cells. The data suggest that MGSA binding to the class II IL-8 receptor is associated with tyrosine phosphorylation of p130/cas. The data also suggest that p130 and the cas antigen are the same protein.

Chemokines represent a family of chemotactic proteins which bind to specific receptors on neutrophils, lymphocytes, monocytes, and a number of other cell types, to effect a chemotactic response and promote cell migration (Kelvin et al., 1993; Baggiolini et al., 1994). The chemokine α subfamily comprises the CXC group of chemokines which largely affect the movement of neutrophils, lymphocytes, and melanoma cells. Included in this group are MGSA[1] or GRO, interleukin-8, (IL-8), neutrophil activating protein-2, platelet activating factor-4, and γ-interferon inducible protein-10 (Baggiolini et al., 1994). The proteins in the chemokine β or CC subfamily [macrophage inflammatory protein 1α and β, regulated upon activation−normal T cell expressed and secreted (RANTES), and monocyte chemotactic protein-1] are primarily chemotactic for monocytes (Baggiolini et al., 1994). Receptors for both subfamilies have been identified and demonstrated to be promiscuous for a number of members of each subfamily, but the α/β chemokines do not bind to receptors across the subfamily lines (Murphy, 1994). Of particular note here, the class I IL-8 receptor binds IL-8 with high affinity while exhibiting little affinity for MGSA, whereas the class II IL-8 receptor has high affinity for IL-8 and MGSA (Lee et al., 1992; Cerretti et al., 1993). In contrast, there is a receptor on erythrocytes which binds *Plasmodium vivax* as well as members of both chemokine α and β subfamilies (Horuk et al., 1993). Excluding the erythrocyte receptor, ligand binding to these receptors is associated with coupling of G proteins to seven-transmembrane receptors (Holmes et al., 1991; Murphy & Tiffany, 1991; Charo et al., 1994) as well as rapid Ca^{2+} influx (Baggiolini et al., 1994).

A number of seven-transmembrane G-protein-coupled receptors, while lacking protein kinase homology motifs, have been shown to have associated tyrosine kinase activity in response to ligand binding. Included in this group are the bombesin, vasopressin, endothelin, bradykinin, and lysophosphatidic acid (LPA) receptors (Zachary et al., 1991; Leeb-Lundberg & Song, 1991; Rankin & Rozengurt, 1994; Hordijk et al., 1994; Seufferlein & Rozengurt, 1994; Leeb-Lundberg et al., 1994). Despite the diversity of these mitogens and their receptors, several common proteins are phosphorylated on tyrosine in response to ligand binding, including p125FAK, p70 paxillin, and the src-associated substrate p130 (Kanner et al., 1991). Members of the src family of tyrosine kinases also phosphorylate these proteins (Kanner et al., 1991; Cobb et al., 1994; Sakai et al., 1994) and thus have been implicated in signaling downstream from the G-protein-coupled receptors.

In addition to the tyrosine phosphorylation of p125-focal adhesion kinase (FAK), paxillin, and p130, a number of other

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[1] Abbreviations: bFGF, basic fibroblast growth factor; Cas, crk-associated substrate; CEF, chick embryo fibroblasts; DMEM, Dulbecco's modified Eagle's medium; FAK, focal adhesion kinase; FBS, fetal bovine serum; fMLP, formylmethionylleucylphenylalanine; GAP, GTP-associated protein; IL-8, interleukin-8; LPA, lysophosphatidic acid; MAP-K, mitogen activated protein kinase; MEM, minimal essential medium; MGSA, melanoma growth stimulatory activity; PC12, pheochromocytoma cell line 12; $PTPD_1$, protein tyrosine phosphatase D_1; RIPA, radioimmunoprecipitation assay.

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proteins undergo either serine/threonine or tyrosine phosphorylation subsequent to activation of seven-transmembrane G-protein-coupled receptors. Stimulation of the LPA receptor induced ras activation and mitogen activated protein (MAP) kinase tyrosine phosphorylation, and these events were pertussis toxin and staurosporine (1 μM) sensitive (Hordijk et al., 1994). Moreover, ligand stimulation of another seven-transmembrane G-protein-coupled receptor, the formylmethionylleucylphenylalanine (fMLP) receptor, resulted in activation of MAP kinase, which was accompanied by the serine/threonine phosphorylation of MAP kinase. The effect of fMLP on MAP kinase was blocked by antagonists of protein kinase C or tyrosine kinases (Grinstein et al., 1994). Likewise, the G-protein-coupled receptor for thrombin induces tyrosine phosphorylation of a number of substrates through multiple pathways (Weiss & Nuccitelli, 1992; Weiss & Maduri, 1993; Offermanns et al., 1993).

Recently, we have demonstrated that the class II IL-8 receptor itself becomes phosphorylated on Ser residues in response to MGSA (Mueller et al., 1994) in a variety of non-hematopoietic cell lines which were stably transfected to overexpress this receptor. We have also demonstrated that MGSA treatment of membrane preparations from human term placenta results in enhanced phosphorylation of a number of proteins, some of which are tyrosine phosphorylated (Cheng et al., 1992). IL-8, also a member of the chemokine α subfamily which can bind to the same receptor as MGSA, has been demonstrated to stimulate the tyrosine phosphorylation of MAP kinase in neutrophils (Van Lint et al., 1993; Thompson et al., 1994). The current model is that in addition to the activation of a serine kinase(s) in response to chemokine α binding to receptors, nonreceptor tyrosine kinases and/or dual specificity kinases are also activated downstream of receptor binding. To better characterize the phosphorylation events induced by MGSA in nonhematopoietic cells, we examined the response to MGSA in the clonal 3ASubE P-3 human placental cell line overexpressing the class II IL-8 receptor (Mueller et al., 1994). Work described herein demonstrates that MGSA binding to the class II IL-8 receptor results in tyrosine phosphorylation of a 130 kDa protein and a 70 kDa protein recognized by antiserum to the src substrate p130 and antibody to the cas protein, also described as a src substrate (Kanner et al., 1990; Sakai et al., 1994).

EXPERIMENTAL PROCEDURES

Methods

Cell Culture. The 3ASubE placental cell line was purchased from ATCC and cultured in miminal essential medium supplemented with 5% fetal bovine serum and penicillin/streptomycin and maintained at 37 °C. Stock cultures of 293 cells were cultured in 5% FBS/DMEM. Prior to experiments with 293 cells, the medium was replaced with 1% FBS/DMEM and cells were cultured 24 h at 37 °C prior to a second medium replacement with 0.25% FBS/DMEM for an additional 6 h at 37 °C. Under these conditions, 293 cells remained viable and loosely attached. Culture medium for T_2 poly IL-8 class II receptor stable transfects and 3ASubE stable transfectants included 400 μg/mL G418.

3ASubE stable transfectants overexpressing the class II IL-8 receptor (3ASubE P-3) or the cells transfected with the pRc/CMV vector alone (3ASubE P-1) were maintained in MEM + 400 μg/mL G-418 and 5% FBS as previously described (Mueller et al., 1994). Cells were cultured at 37 °C in a water-jacketed CO_2 incubator in a 5% CO_2/95% air atmosphere. Prior to use, medium was removed, and cells were serum-starved for 40 h at 37 °C in serum-free DMEM. Prior to MGSA stimulation, starvation medium was replaced with fresh serum-free DMEM. After temperature equilibration, 5 nM MGSA was added to cells in vehicle containing 4 mM HCl and 0.25 mg/mL BSA for times indicated in the Figures. Nontreated controls received vehicle alone.

Preparation of Whole Cell Lysates, Membranes, and Cytosolic Fractions. (A) Whole Cell Lysates. After cells were stimulated with MGSA or carrier buffer, medium was carefully aspirated, cells were washed once with 10 mL of ice-cold isotonic saline, and cells were immediately lysed with ice-cold 1% Triton X-100 Tris-buffered saline (TBS), pH 7.5, containing 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 10 mM NaF, and 10 μg/mL each of aprotinin and leupeptin. In some instances, this nondissociating buffer was supplemented with 0.5% deoxycholate and 0.1% SDS (RIPA). Cells were scraped, and lysates were incubated on ice 15 min and microfuged 10 min at 4 °C. Clear supernatants were stored frozen at −80 °C.

(B) Membrane and Cytosolic Fractions. 3ASubE P-3 cells were stimulated for 2 min at 37 °C with or without 5 nM MGSA. Medium was removed, and cells were washed once with 10 mL of ice-cold TBS, pH 7.5, and immediately lysed by scraping in 2.5 mL of hypotonic lysis buffer (Lin et al., 1991) per 15 cm dish. Lysis buffer contained 10 mM Tris-Cl, pH 7.5, 50 mM mannitol, 1 mM PMSF, 1 mM Na_3VO_4, and 10 μg/mL each of aprotinin and leupeptin. Lysed cells were vesiculated with 8 passes through a 23 gauge needle and adjusted to 10 mM $CaCl_2$. After microfuging for 1 min, the supernatant was centrifuged at 100000*g* for 1 h at 2 °C. The resulting supernatant/cytosol was adjusted to 11 mM EDTA and stored frozen at −80 °C. The 100000*g* pellet representing the membrane fraction was washed twice with 10 mM Tris-HCl, pH 7.5, and stored at −80 °C. In some instances, this membrane was resuspended in 0.5 M NaCl and 10 mM Tris-Cl, pH 7.5, for 0.5 h on ice, repelleted, washed with 10 mM Tris-Cl, pH 7.5, and stored frozen at −80 °C. For anti-phosphotyrosine immunoprecipitation of cytosol, antibody was routinely added in detergent-free solution, and immunoprecipitations were performed as described.

Protein Estimation. Equal protein was loaded on all gels (whole cell lysates) or for immunoprecipitations. Protein was estimated with BCA (Pierce) using BSA as the standard.

Immunoprecipitation. Anti-phosphotyrosine immunoprecipitations were routinely performed in Triton X-100-containing buffer using 1 μg of Zymed rabbit polyclonal or in some instances 1 μg of ICN PY20 antibody per 50−100 μg of protein lysate. After a 2−3 h incubation on ice, 15−30 μL of protein A/G−agarose (Pierce) was added for 1 h. Immunoprecipitates were pelleted by centrifugation, washed four times with ice-cold lysis buffer, and denatured in 0.5% SDS/10% β-mercaptoethanol-containing Laemmli sample buffer. Immunoprecipitations with antibodies to FAK and paxillin were performed as described above except that 150−500 μg protein and RIPA buffer were used. Immunoprecipitations with antibody to p130 using 4 μL of ascites fluid

for 500 μg of protein were performed as for the phosphotyrosine immunoprecipitates, but rabbit anti-mouse IgM was used as a sandwich. Immunoprecipitations with anti-cas-2 antibody required 1−2 μL of serum per 500 μg of protein.

Western Blot Analysis. Western blot analyses of whole cell lysates or immunoprecipitates were performed according to our standard procedures (Mueller et al., 1994). Briefly, proteins were separated by electrophoresis through 8.75% Laemmli gels (Laemmli, 1970) and then transferred to PVDF membranes. Membranes were blocked in 5% milk powder/phosphate-buffered saline (PBS) or Tris-buffered saline (TBS), pH 7.5, 1 mM EDTA, and 1 mM Na_3VO_4 for 2 h at 25 °C or overnight at 4 °C. Blots were incubated in primary antibody containing 2.5% milk powder and the appropriate EDTA/vanadate buffer for 2−3 h at 25 °C, washed four times for 4 min/wash with 10 mL of the same buffer without EDTA or vanadate, pH 7.5 at 25 °C, and then incubated with alkaline phosphatase-conjugated secondary antibody (1:1000 dilution) or ^{125}I-protein A (5×10^5 cpm/mL) in 10 mM Tris-buffered saline, pH 7.5, containing 2.5% milk powder. After four consecutive TBS pH 7.5 washes, the blots were developed with bromochloroindolyl phosphate/nitroblue tetrazolium (Figures 1, 2A, and 3−5) or ^{125}I-protein A (Figures 1A, 2B, 4, and 6) followed by autoradiography. When mouse monoclonal antibodies were used, the immunological development protocol included incubation with a rabbit anti-mouse antibody. Generally, 1 μg of mouse Ab was captured with 5 μg of rabbit anti-mouse antibody for 1 h at 4 °C (immunoprecipitation) or 1 h at 25 °C (protein A blots).

Data Analysis. Intensity of bands on Western blots or autoradiograms was determined by scanning using an Epson ES 1200C densitometric scanner and the Adobe Photoshop software. Digitized images were subsequently scanned using the Molecular Dynamics phosphoimager. Volumes for paired observations were integrated and normalized to a value of one for the control of each pair. Statistical analyses (Wilcoxon rank sum test) were performed using a null hypothesis that median unstimulated values are equal to the median for MGSA stimulated values.

Materials

Cells. The 3ASubE placental and 293 cell lines were obtained from ATCC. The 3ASubE cell line carries a temperature-sensitive SV40 tsA30 virus which is expressed at 37 °C but not at the restrictive temperature (40 °C) (Chou, 1978). The parental cell line does not express detectable levels of the class I or class II IL-8 receptor, based upon analysis by Northern blot and RT-PCR, or Western blot analysis using antibody developed to the human class II receptor (Mueller et al., 1994). ^{125}I-MGSA binding assays detect no specific binding of this ligand to parental 3ASubE placental cells at 37 °C. The stably transfected clonal cell lines 3ASubE P-3 and 293 T_2 expressing the IL-8 receptor B have been previously described (Mueller et al., 1994).

Reagents. Anti-phosphotyrosine antibodies were from Zymed (rabbit polyclonal), Transduction labs (PY20), and ICN (PY20). Anti-paxillin was from Transduction Labs. Anti-FAK was a gift from Dr. Steve Hanks (Vanderbilt University) or Dr. Tom Parsons (University of Virginia). Anti-p130 (Kanner et al., 1990) was also a gift from Dr. Parsons. Anti-cas-2 was from Dr. Hisamaru Hirai (University of Tokyo) (Sakai et al., 1994), and anti-$PTPD_1$ was from Dr. Reiner Lammers (Max Planck Institut fur Biochemie) (Moller et al., 1994). Goat or rabbit alkaline phosphatase-conjugated secondary antibody was from Sigma. Anti-mouse antibodies were from Sigma and Zymed. Protein A was radioiodinated using the chloramine T method (Hunter & Greenwood, 1962). MGSA was a gift from R & D Systems. All other chemicals used were reagent grade.

RESULTS

MGSA Induces Tyrosine Phosphorylation in Class II IL-8 R Stable Transfectants But Not in Vector Controls. We have previously shown that MGSA stimulates serine phosphorylation of the class II IL-8 receptor expressed in the stably transfected 3ASubE P-3 and 293 T_2 poly cells (Mueller et al., 1994). This stimulation was concentration and time dependent with maximal phosphorylation occurring after a 5 min treatment with 5 nM MGSA. In prior work (Cheng et al., 1992) we have also demonstrated that proteins in membrane preparations from human placenta were phosphorylated on tyrosine in response to MGSA. Therefore, we chose to further examine MGSA induced tyrosine phosphorylated substrates in the clonally selected, stably transfected placental cell line termed 3ASubE P-3. Initially, to determine whether MGSA-enhanced tyrosine phosphorylation of proteins in the 3ASubE P-3 cells was mediated through the class II receptor and not some endogenously expressed receptor for MGSA in the 3ASubE cells, we compared the MGSA-enhanced phosphorylation in 3ASubE P-3 cells to that from a clone stably transfected with the parental expression vector. 3ASubE P-3 and vector controls (3ASubE P-1) were either stimulated or not stimulated with MGSA (5 nM) for 1 or 2 min. Western blot analyses using anti-phosphotyrosine antibodies revealed proteins in the 66−70 and 116−130 kDa ranges which appeared to be constitutively phosphorylated on tyrosine residues in both cell types (data not shown). The 3ASubE P-3 cells exhibited enhanced tyrosine phosphorylation of the 130 kDa proteins in response to MGSA; however, there was no response to MGSA in the P-1 vector control cells (data not shown). These results suggest that the MGSA-enhanced tyrosine phosphorylation events are being mediated through the IL-8 receptor B (IL-8 RB). Results similar to those above were obtained for the 3ASubE P-3 cells when anti-phosphotyrosine immunoprecipitations were performed and subjected to Western analysis for evaluation of tyrosine phosphorylation (data not shown).

In order to evaluate the time course over which the tyrosine phosphorylation events were sustained, 3ASubE P-3 cells were treated with MGSA (5 nM) for increasing periods of time (1−30 min), and whole cell lysates were prepared and subjected to Western blot analysis using anti-phosphotyrosine antibodies. As shown in Figure 1, for 3ASubE P-3 cells, the ~130 kDa protein showed enhanced tyrosine phosphorylation after MGSA stimulation. Tyrosine phosphorylation of the 130 kDa band appeared to be maximal by 1−2 min based upon alkaline phosphatase detection of Western blotting and returned to the basal level after 5 min. Similar results were obtained when the anti-phosphotyrosine immunoblot was developed with ^{125}I-protein A (data not shown). MGSA-enhanced tyrosine phosphorylation was demonstrable for 3ASubE P-3 cells using either the ICN PY20 phosphotyrosine antibody shown above (Figure 1A) or Zymed antibody (Figure 1B). When the densities of the tyrosine phosphorylated proteins observed at the 1 and 2 min time

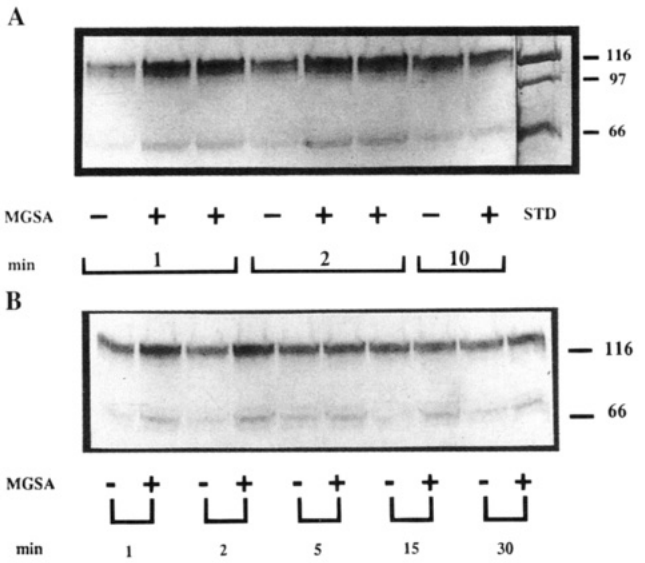

FIGURE 1: Time course of MGSA-dependent phosphorylation of 130 and 70 kDa proteins in 3ASubE P-3 cells. Whole cell lysates (100 μg) prepared from 3ASubE P-3 cells stimulated with or without 40 ng/mL MGSA for the times indicated were subjected to Western blot analysis, and blots were developed with anti-phosphotyrosine antibody with alkaline phosphatase detection as described in Methods. In (A) the blot was incubated with ICNαPY20 antibody prior to development. Molecular mass standards in lane 10 are galactosidase, phosphorylase, and bovine serum albumin. Molecular masses are indicated in kDa. In (B) the blot was incubated with Zymed polyclonal anti-phosphotyrosine antibody prior to development. Each experiment was repeated four times for both (A) and (B), giving a total $n = 8$, with similar results each time.

points were determined by scanning, the results revealed that, at 1 min, the mean density of the 130 kDa band was 2 $\pm$ 0.24-fold greater than that observed in the unstimulated cells ($n = 8$, $\alpha = 0.008$ based on the Wilcoxon rank sum test). At the 2 min time point the density of the 130 kDa phosphotyrosine band in the MGSA stimulated cells from combined studies described in Figure 1, panels A and B, was 1.79 $\pm$ 0.15-fold greater than in the unstimulated cells ($n = 8$, $\alpha = 0.008$). All other time points (5, 10, 15, and 30 min) in Figure 1 did not show significant differences in tyrosine phosphorylation of the 130 kDa protein in treated and untreated cell lysates. The 70 kDa protein appeared as a doublet, with the upper band of the doublet exhibiting the greater level of phosphorylation on tyrosine. In lysates of whole cells, we were able to demonstrate a statistically significant difference in the ligand-enhanced phosphorylation of the 70 kDa protein at both the 1 and 2 min time points. After 1 min stimulation, the fold increase in tyrosine phosphorylation of the 70 kDa band/dimer in response to MGSA was 1.828 $\pm$ 0.26 ($n = 8$, $\alpha = 0.008$), while after 2 min stimulation, the 70 kDa band in MGSA-treated samples showed a density 2.063 $\pm$ 0.24-fold greater than of control ($n = 8$, $\alpha = 0.008$).

To determine if the tyrosine phosphorylation observed in response to MGSA treatment of the 3ASubE P-3 cells was unique to this cell type or whether it could be observed in other cell lines, a similar study was conducted using the human 293 fibroblasts transfected and selected for stable expression of the class II IL-8 receptor (293 T_2 poly). The 293 T_2 poly cells were treated with MGSA for increasing periods of time, and whole cell lysates (WCL) were prepared and analyzed as described above for the 3ASubE P-3 cells.

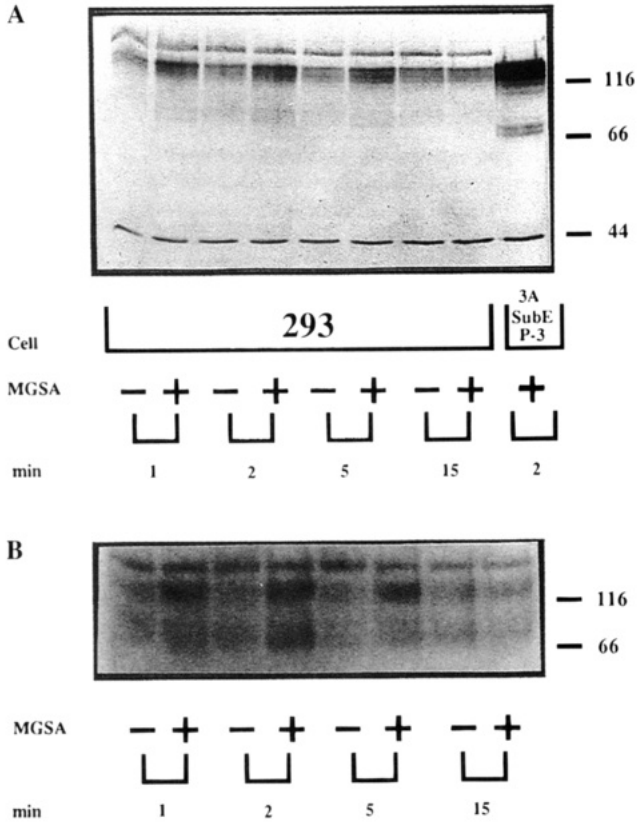

FIGURE 2: Time course of MGSA-dependent tyrosine phosphorylation of 130 and 70 kDa proteins in 293 T_2 polyclonal cells. Whole cell lysates prepared from 293T_2 poly or 3ASubE P-3 cells treated with or without 40 ng/mL MGSA for the times indicated were analyzed by Zymed anti-phosphotyrosine, alkaline phosphatase Western blotting (A) or by ^{125}I-protein A Western blotting (B) as described in Methods. The blot in the upper panel was reblocked and immunostained for MAP kinase (ERK-2, 42 kDa) to demonstrate equal loading. The experiment in the (A) was repeated four times with similar results each time.

As was demonstrated for the 3ASubE P-3 cells, MGSA treatment of 293 T_2 poly cells also resulted in the enhanced tyrosine phosphorylation of a protein of 130 kDa (Figure 2). The kinetics of the tyrosine phosphorylation of the 130 kDa protein in the 293 T_2 poly cells was similar to that observed in the 3ASubE P-3 cells except that enhanced tyrosine phosphorylation of the 130 kDa band was still elevated approximately 2-fold at 5 min treatment in the 293 cells but not in 3ASubE P-3 cells. Furthermore, in 293 cells the 70 kDa protein was maximally tyrosine phosphorylated by 2 min and the level of phosphotyrosine declined by 15 min (Figure 2). Equal loading for 293 cell lysates was demonstrated by anti-MAP kinase immunostaining (Figure 2A, 42 kDa band). Similar results were obtained when the Western blot was developed with either ^{125}I-protein A (Figure 2B) or the alkaline phosphatase (Figure 2A) conjugated secondary antibody/alkaline phosphatase substrate detection system. Evaluation of the tyrosine phosphorylation of the 130 kDa band from a series of four experiments (recorded as the mean ratio of the density $\pm$ standard deviation for treated versus untreated controls) revealed that MGSA stimulated a 2.179 $\pm$ 0.34-, 2.2270 $\pm$ 0.24-, 2.172 $\pm$ 0.25-, and 0.94 $\pm$ 0.04-fold increase for the 1, 2, 5, and 15 min time periods, respectively. Using the Wilcoxon rank sum test, these means were significant at the α <0.01 level for the 130 kDa band at the 1, 2, and 5 min time points only. Though the tyrosine phosphorylation of the 70 kDa protein

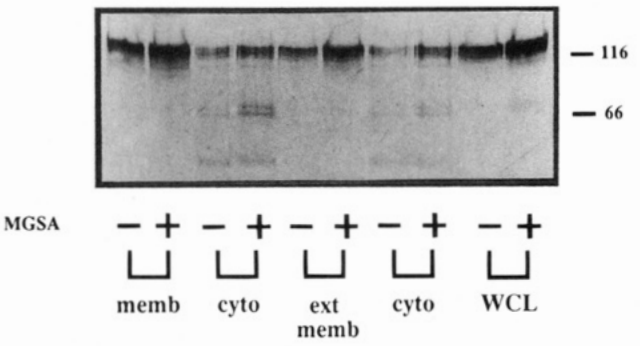

FIGURE 3: Distribution of the 130 and 70 kDa phosphotyrosine-containing proteins in membrane and cytosolic components of 3ASubE P-3 cells. Control or MGSA-treated (2 min) cells were fractionated as described in Methods into membrane (memb) and cytosolic (cyto) components. Membrane (50 μg of protein) was left untreated or extracted (ext memb) with high and low ionic strength. Whole cell lysate (WCL) (100 μg) is shown for comparison. Duplicate anti-phosphotyrosine immunoprecipitations of cytosol (200 μg of protein) were performed. Immunodetection was by alkaline phosphatase-conjugated secondary antibody as described in Methods. The experiment was repeated twice with similar results.

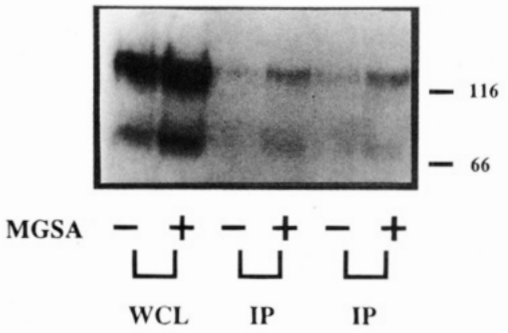

FIGURE 4: Immunoprecipitation of MGSA-dependent 130 and 70 kDa phosphotyrosine-containing proteins using anti-p130 antibody. 3ASubE P-3 cells were stimulated for 2 min without or with MGSA. Triton X-100-containing lysates (500 μg of protein) were immunoprecipitated (IP) using the src substrate anti-p130 antibody (4 μL), and Western blotted with anti-phosphotyrosine antibody, and phosphotyrosine-containing proteins were detected with ^{125}I-protein A as described in Methods. Whole cell lysate (WCL) (100 μg of protein) is shown for comparison. The experiment described here was repeated five times with similar results each time.

in the 293 T_2 poly cells was not as apparent as in the 3ASubE P-3 cells, the approximate 2-fold increase in the basal phosphorylation of this band in response to MGSA was maximal at 2 min for the four experiments examined.

Localization of the 130 and 70 kDa MGSA-Enhanced Tyrosine Phosphorylated Proteins to Membrane and Cytosolic Compartments, Respectively. To determine where the 130 and 70 kDa proteins resided in the cell, membrane and cytosolic fractions were prepared and analyzed from 3ASubE P-3 cells. 3ASubE P-3 cells were stimulated for 2 min with or without 5 nM MGSA, hypotonically lysed, vesiculated, and fractionated to yield membrane and cytosol fractions. Crude membranes were either extracted with cycles of low and high ionic strength buffer or not extracted. Equal amounts of membrane proteins were subjected to Western blot analysis using phosphotyrosine antibodies. The cytosol was immunoprecipitated with anti-phosphotyrosine antibody (Zymed) and subsequently analyzed by Western blot. As demonstrated in Figure 3, the 70 kDa phosphotyrosine protein was found exclusively in the cytosolic immunoprecipitates (compare lanes 3, 4, 7, 8 with 1, 2, 5, 6). The band at ~55 kDa in the cytosolic lanes is the anti-phosphotyrosine antibody heavy chain used for immunoprecipitation. In contrast, the 130 kDa protein was distributed between cytosolic and membrane fractions. The 130 kDa phosphoprotein appears as a broad band which may be comprised of two or more proteins. Alternatively, the broad band may represent alternate forms of the same protein. These data show that MGSA enhances the tyrosine phosphorylation of membrane-bound and cytosolic proteins in these cells overexpressing the class II IL-8 receptor.

Identification of the 130 and 70 kDa MGSA-Enhanced Tyrosine Phosphoproteins. Numerous seven-transmembrane G-protein-coupled receptors induce the tyrosine phosphorylation of FAK (125 kDa) and paxillin (68 kDa) upon ligand binding (Leeb-Lundberg et al., 1994; Hordijk et al., 1994; Seufferlein & Rozengurt, 1994). To explore the hypothesis that MGSA enhances the tyrosine phosphorylation of FAK and paxillin, we immunoprecipitated lysates prepared from control or MGSA-treated 3ASubE cells with antibodies to FAK and/or paxillin, transblotted, and probed with phosphotyrosine antibodies. We did not detect any differences in tyrosine phosphorylation of FAK in response to MGSA, though there was evidence for a faint tyrosine-phosphorylated form of FAK in anti-phosphotyrosine immunoprecipititates of whole cell lysates from 3ASubE P-3 cells in both MGSA-treated and untreated cells. We also did not observe any MGSA-enhanced tyrosine phosphorylation of paxillin (data not shown).

A number of proteins associated with focal adhesions are phosphorylated on tyrosine in src-transfected cells (Kanner et al., 1990). Antibodies have been developed to one of these proteins, referred to as pp130 (Kanner et al., 1990). When whole cell lysates from 3ASubE P-3 cells were immunoprecipitated with the p130 antibody (Kanner et al., 1990) and the immunoprecipitates were electrophoresed, transblotted, and probed with the Zymed anti-phosphotyrosine antibody, we were able to detect a prominent tyrosine-phosphorylated band which showed enhanced tyrosine phosphorylation after MGSA treatment (Figure 4, lanes 4 and 6). MGSA stimulated approximately a 3-fold increase (2.91 $\pm$ 0.29 with a range from 2.4 to 3.3, $n = 5$) in the tyrosine phosphorylation of p130 as quantitated by phosphoimage analysis (Molecular Dynamics). The 70 kDa protein which was tyrosine phosphorylated in response to MGSA treatment (2.99 $\pm$ 0.20-fold stimulation, with a range from 2.76 to 3.13, $n = 5$) in the 3ASubE P-3 cells was also apparent on the Western blot of the p130 immunoprecipitates, suggesting that this protein either is immunologically similar to the src substrate p130 or alternatively is a coimmunoprecipitating protein. Control immunoprecipitations using an IgM antibody to CD15 did not produce tyrosine-phosphorylated immunoprecipitated proteins (data not shown). These data suggest that the major proteins showing enhanced tyrosine phosphorylation in response to MGSA are immunologically similar to the src substrate p130 (Kanner et al., 1991; Cobb et al., 1994). In the same experiment, the tyrosine phosphorylation of the lysates from whole cells (without immunoprecipitation using pp130 antibody) revealed that MGSA stimulated a 1.74 $\pm$ 0.10-fold increase in the density of the 130 kDa band and a 1.83 $\pm$ 0.124-fold increase in the density of the 70 kDa band. The basal level of phosphorylation in the whole cell lysates pictured in this experiment was much greater than was observed after immunoprecipitation, sug-

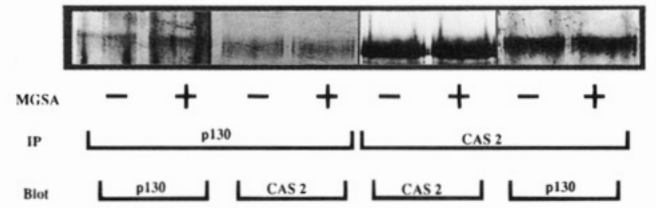

FIGURE 5: Immunological cross-reactivity of immunoprecipitated 130 kDa MGSA-dependent tyrosine phosphoprotein using anti-p130 and anti-cas-2 antibodies. 3ASubE P-3 cells were stimulated for 2 min with or without MGSA (5 nM). Triton X-100 lysates (500 μg of protein) were immunoprecipitated using either anti-p130 (4 μL) or anti-cas-2 (2 μL) antibodies as indicated. Immunoprecipitates were then Western blotted with the homologous or heterologous antiserum and detected using alkaline phosphatase-conjugated secondary antibody as described in Methods. These experiments were repeated three times with similar results.

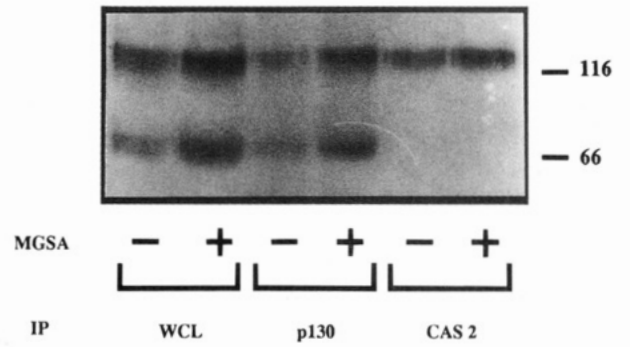

FIGURE 6: The 130 kDa MGSA-dependent protein immunoprecipitated using anti-p130 and anti-cas-2 antibodies contains phosphotyrosine. 3ASubE P-3 cells were stimulated for 2 min without or with MGSA. Triton X-100 lysates (400 μg of protein) were immunoprecipitated using anti-p130 antibody (3 μL) or anti-cas-2 (1 μL) antibody. Whole cell lysate (WCL, 70 μg of protein) is shown for comparison. After Western blotting with anti-phosphotyrosine antibody, phosphotyrosine-containing proteins were detected with ^{125}I-protein A. The experiment was repeated twice with anti p130 and three times using anti-cas with similar results.

gesting that p130 does not represent the only 130 kDa tyrosine-phosphorylated protein in these lysates. Second, there is some variability from experiment to experiment with the efficiency of the immunoprecipitation from the whole cell lysates. The combination of these two factors can contribute to differences in the percentage of the tyrosine-phosphorylated protein precipitated with the p130 antibody, as is illustrated in Figures 3−6. However, the observation that the fold induction in tyrosine phosphorylation after MGSA treatment is greater after immunoprecipitation using the antibody to p130 suggests that p130 is a major target for the MGSA-stimulated tyrosine phosphorylations.

Immunological Characterization of p130. Recently, two src substrate proteins in the 130 kDa molecular mass range have been cloned. These 130 kDa proteins were identified as cas or crk-associated substrate (Sakai et al., 1994) and protein tyrosine phosphatase D_1 ($PTPD_1$), a phosphatase with Ezrin-like domains (Moller et al., 1994). According to the observation that the p130 antiserum used here was derived from src coimmunoprecipitation-purified protein (Kanner et al., 1990), we asked whether these polypeptides share common epitopes. To investigate this, we immunoprecipitated with anti-p130 (Kanner et al., 1990) or anti-cas-2 (Sakai et al., 1994) antiserum and subsequently examined Western blots with homologous or non-homologous antiserum. Figure 5 shows control and MGSA-stimulated 3ASubE P-3 lysates immunoprecipitated with anti-p130 or anti-cas-2 as indicated. As shown in Figure 5, both antisera cross-reacted, suggesting common epitopes. The difference in the electrophoretic mobilities of the immunoreactive bands is consistent with the properties of the anti-cas-2 antibody (see Discussion section). In contrast, comparison of anti-p130 and anti-$PTPD_1$ immunoreactivity showed no cross-reactivity (data not shown).

To further confirm that the anti-p130, anti-cas antibodies recognized the 130 kDa protein which was phosphorylated in response to MGSA, lysates were prepared from 3ASubE P-3 cells which had been treated with MGSA and the lysates were immunoprecipitated with anti-p130 or anti-cas-2 antiserum followed by anti-phosphotyrosine Western blotting. As shown in Figure 6, anti-p130 immunoprecipitates showed MGSA-dependent increases in tyrosine phosphorylation (2.54-fold over control, $n = 2$) for the 130 kDa protein, as did the anti-cas-2 immunoprecipitates (2.34-fold over control, $n = 3$). Only the p130 immunoprecipitates, however, showed the 70 kDa immunoreactive protein. These data suggest that either the 70 kDa protein is a breakdown product of p130 which is not recognized by the cas-2 peptide antibody or it is associated with proteins coimmunoprecipitated with p130 antibody, but not with the cas-2 antibody. To our knowledge, this is the first report demonstrating the antigenic similarity of the 130 kDa protein recognized by the p130 and cas antibodies.

DISCUSSION

Data from anti-phosphotyrosine immunoprecipitation experiments of MGSA treated cells expressing the class II IL-8 receptor support the concept that ligand-induced tyrosine as well as serine phosphorylation events can be mediated through the class II IL-8 receptor. MGSA (5 nM) enhanced the tyrosine phosphorylation of a 130 kDa protein and the peak of tyrosine phosphorylation occurred at 2 min, prior to the peak time for serine phosphorylation of the class II receptor, which occurred at 5 min (Mueller et al., 1994). These phosphotyrosine-containing proteins were evident in both 3ASubE P-3 placental cells and 293 T_2 polyclonal kidney epithelial cells stably transfected with the class II IL-8 receptor. Since these two cell lines are immortalized by two independent means (SV40 or EIA proteins, respectively), it is unlikely that the phosphorylations are occurring as a result of interaction with the immortalizing protein rather than directly through receptor activation. Further support for this interpretation comes from the observation that the MGSA-enhanced phosphorylations were not detected in vector control stable transfectant cell lines but were observed when receptor expressing 3ASubE P-3 cells were grown at the nonpermissive temperature (40 °C) for the SV40 tsA30 mutant (unpublished observations). The number and size of the proteins phosphorylated on tyrosine were different than observed in whole placental tissue using the in vitro phosphorylation assay (Cheng et al., 1992). The 3ASubE P-3 placental cell line is derived from the syncytiotrophoblast and does not show the same phosphorylation pattern using the in vivo phosphorylation assay described here as compared to the in vitro phosphorylation assay of human term placental tissue which includes endothelial cells, trophoblast cells, cytotrophoblasts, and blood cells of all types, as well as other cell types from mesodermal and ectodermal derivatives. However, the ability to observe a similar phosphorylation

pattern in both the 3ASubE P-3 clones and the 293 T_2 poly clones expressing the IL-8 receptor B suggests that these tyrosine phosphorylation events are mediated through the IL-8 receptor B in response to MGSA and may be associated with biological activity in response to this chemokine.

High apparent basal phosphorylation of the 130/70 kDa proteins in conjunction with comigrating constitutively tyrosine phosphorylated proteins in the electrophoretic gels severely hampered study of MGSA-dependent effects during the initial portion of this investigation. Anti-phosphotyrosine blots of unfractionated cell lysates reproducibly showed a modest 2-fold difference between control and MGSA-stimulated 130/70 kDa proteins. The endogenous tyrosine phsophorylation could be associated with inability to totally arrest and synchronize these cells prior to MGSA treatment. Moreover, it is probable that other residual serum factors or even endogenously produced MGSA may contribute to the basal level of tyrosine phosphorylation of these cells. Other proteins found in focal adhesions show high levels of endogenous phosphorylation when grown in serum and on the appropriate substratum (Kanner et al,, 1991; Cobb et al., 1994).

Attempts to identify the 130 protein from MGSA-stimulated or control 3ASubE P-3 or 293 T_2 cells revealed these tyrosine-phosphorylated proteins could be immunoprecipitated with anti-p130 (Kanner et al., 1990). The p130 protein which shows enhanced phosphorylation in response to MGSA was originally identified in activated v-src coimmunoprecipitation complexes that required intact src SH_2 domains (Kanner et al., 1991), suggesting that cellular nonreceptor tyrosine kinases may be involved in MGSA stimulated signal transduction. Recently, p125(FAK) has also been shown to stably associate with activated p60src and p59fyn (Cobb et al., 1994). Moreover, two additional 130 kDa proteins, $PTPD_1$ (Moller et al., 1994) and cas (Sakai et al., 1994), were cloned and shown to be associated with either c-src (Moller et al., 1994), v-crk, or v-src (Sakai et al., 1994), presumably via SH_2/SH_3 domains of v-src. Comparison of immunological cross-reactivity between immunoprecipitates of 3ASubE P-3 lysates using antisera against p130 (Kanner et al., 1990) and cas-2 (Sakai et al., 1994) showed common epitopes. It should be noted that the cas-2 antibody was raised against a bacterial fusion protein containing a portion of the substrate region (Sakai et al., 1994) where SH_2 domain interactions would be anticipated to occur. In contrast, p130 (Kanner et al., 1990) was generated against an intact phosphotyrosine-containing polypeptide that coimmunoprecipitated with v-src. Thus, the data suggest common epitopes in the 130 kDa MGSA-stimulated protein recognized by p130 and cas-2 antisera. However, the p130 antibody would be expected to recognize predominately the slower migrating tyrosine-phosphorylated form of p130/cas. In contrast, a similar series of experiments to compare the effects of MGSA on the tyrosine phosphorylation of p130 and the ezrin-like protein tyrosine phosphatase $PTPD_1$ suggested that p130 and $PTPD_1$ do not have common epitopes.

Several questions remain unanswered if indeed cas and pp130 are the same. The first question is are all of the tyrosine phosphorylated moieties accounted for by the p130 and/or cas-2 antigens? Clearly, at the 2 min time point the ratios comparing the intensities of immunoreactivity associated with tyrosine phosphorylation in response to MGSA are higher after cas or p130 immunoprecipitation of whole cell lysates. The ratio of tyrosine phosphorylation in MGSA-treated versus untreated whole cell lysates was 1.79 ± 0.15 as compared to 2.91 ± 0.29, $n = 5$, for the immunoprecipitates of the 130 kDa protein, suggesting that p130 or cas antigen accounts for a significant fraction of the protein undergoing ligand-dependent tyrosine phosphorylation. However, constitutive tyrosine phosphorylation(s) in the whole cell lysates appears to mask the effect of exogenous MGSA on p130 tyrosine phosphorylation. MGSA-enhanced phosphorylations were more obvious after cell fractionation when it became apparent that the tyrosine-phosphorylated 130 kDa protein was membrane bound and that the 70 kDa protein was cytosolic. We cannot rule out the possibility that some of the endogenous phosphorylation observed in the whole cell lysates is on proteins other than p130/cas. Additionally, it should be noted that though there is some experimental variability in the immunoprecipitation efficiency as seen in the relative intensity of the 130 and 70 kDa bands in Figure 4 versus 6, the ratios of the intensities of stimulated versus unstimulated phosphorylation densities were independent of this variability.

Another question raised by these data is the one of whether the 70 kDa protein doublet is a degradation product of p130 which fails to be immunoprecipitated by the cas-2 antibody. Since the time course experiments never indicated a shift in intensity from the 130 to the 70 kDa proteins using either p130 or cas-2 antibody by immunoblot analysis, this does not seem likely. If indeed the 70 kDa protein doublet is a separate protein(s) which is antigenically similar to p130 and cas, then why does the cas-2 antibody not precipitate the 70 kDa protein? One explanation is that the 70 kDa protein could be generated from a variant mRNA transcript rather than proteolytic degradation and the cas antibody might not recognize the tyrosine-phosphorylated epitopes in p70 which the p130 antibody recognizes.

As with other G-protein-coupled receptors, ligand activation of the class II IL-8 receptor with MGSA results in both serine and tyrosine kinase activity. The receptor itself exhibits marked phosphorylation on serine residues as a result of ligand binding, and in addition, we show here that there are also subtle but consistent changes in the tyrosine phosphorylation of a 130 kDa substrate which is antigenically similar to cas-2. Several other investigators have shown that p130 is phosphorylated on tyrosine residues in response to ligand binding. The phosphorylation of p130 has been studied in association with the signal transduction in response to bradykinin in Swiss 3T3 cells (Leeb-Lundberg et al., 1994). Others have demonstrated p130 to be tyrosine phosphorylated in response to LPA binding to its receptor in Swiss 3T3 cells (Seufferlein & Rozengurt, 1994; Zachary et al., 1991; Rankin & Rozengurt, 1994). Tyrosine phosphorylation of p130 has been demonstrated in PC12 cells and in transformed fibroblasts (Hempstead et al., 1994), in transformed and nontransformed CEF cells (Birge et al., 1993), as well as in association with crk in rat 3Y1 cells (Ogawa et al., 1994). Therefore, the regulation of p130 phosphorylation through seven-transmembrane G-protein-coupled receptors in response to ligands which stimulate cell migration is of great importance. Ligand activation of the seven-transmembrane G-protein-coupled IL-8 receptors is associated with migration of neutrophils, smooth muscle cells, melanoma cells, and keratinocytes (Baggiolini et al.,

1994). Receptor activation is also associated with changes in actin conformation and organization (Westlin et al., 1992). Cas proteins are src substrates which have been implicated to be associated with focal adhesions and the cytoskeleton. Thus it is likely that IL-8 receptor activation by MGSA results in activation of a series of kinases which lead to changes in the cytoskeleton associated with the motility required for chemotaxis.

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