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SURAMIN MODULATES CELLULAR LEVELS OF HEPATOCYTE GROWTH FACTOR RECEPTOR BY INDUCING SHEDDING OF A SOLUBLE FORM

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Abstract—Several growth factor receptors undergo shedding from the cell surface as a result of limited proteolysis via mechanisms that are at present poorly understood. By Western blotting of the conditioned media and cell lysates of several cell lines expressing the hepatocyte growth factor receptor, we found that suramin, a pharmacological agent that inhibits the activity of many growth factors, was able to induce shedding of this receptor. Increased levels of soluble hepatocyte growth factor receptor were observed in the conditioned media of GTL-16, a cell line over-expressing the receptor, as early as ten minutes after initial exposure to the agent, and incubation of this line with 300 μM suramin caused a 50% reduction in cell-associated levels of receptor after 6 hours. Although protein kinase C activation by treatment of cells with phorbol esters has previously been found to stimulate shedding of the hepatocyte growth factor receptor, this hitherto undescribed activity of suramin was not affected by protein kinase C inhibitors. Since shedding represents a possible means of down-modulation of receptor activity, suramin may inhibit the hepatocyte growth factor ligand/receptor system, not only by abrogation of hepatocyte growth factor binding to intact receptor, but also by induction of receptor shedding.

Key words: suramin; HGF; cMet; soluble receptor; proteolysis; growth factor receptor

Suramin, an anti-trypanosomal agent developed in the early years of this century, is able to bind to and inhibit a large number of proteins and enzymes [1]. In recent years it has received much attention following reports of its possible effectiveness as an antitumor agent, a property that might be explained at least in part by its capacity to inhibit receptor binding and/or biological activity of numerous growth factors [2]. Some of the factors inhibited by this drug are heparin-binding, such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) [3–6]. Like heparin, suramin is a polysulfated compound, and since bFGF crystallises with two sulfate ions in the putative heparin-binding region [7, 8], it is tempting to speculate that suramin binds to at least some growth factors at their heparin-binding sites, consequently abrogating binding to receptor. However, suramin is able to inhibit several growth factors that are not heparin-binding, for example, interleukins 1, 2, and 6 (Il-1, IL-2, Il-6), and tumor necrosis factor-α (TNFα) [9–12], so a unique mechanism of inhibition for all factors is as yet unproven, and perhaps unlikely in view of the drug's extremely pluripotent nature.

During the course of studies to characterise inhibition of HGF biological activity and receptor activation by suramin, we observed that moderate concentrations of this agent are able to downregulate cellular levels of the HGF receptor. This receptor, also known as the cMet proto-oncogene, is a hetero-dimeric tyrosine kinase consisting of a transmembrane β-chain of 145 kDa, the intracellular portion of which contains the kinase domain, and a 50 kDa α-chain that is disulphide linked to the extracellular portion of the β-chain [13]. It has previously been found that cells expressing this receptor constitutively shed a soluble form from the cell surface, consisting of the intact α-chain and a C-terminal truncated form of the β-chain, which lacks the kinase and transmembrane domains [14]. The release of a soluble form as the result of limited extracellular proteolytic cleavage has been described for several growth factor receptors in addition to cMet [15]. Although the mechanisms by which receptor shedding occurs are as yet poorly understood, several agents have been found to activate this process. In the case of the HGF, colony-stimulating factor-1, Il-6, and TNFα receptor, for example, it has been demonstrated that activation of protein kinase C by stimulation of cells with phorbol esters results in substantial upregulation of receptor shedding [14, 16–18]. In this report we describe the downmodulation of cellular levels of cMet by suramin, and the concomitant induction in the extracellular milieu of soluble receptor. This is a process that proceeds independently of protein kinase C, and we suggest that it represents a further means by which suramin might modulate growth factor activity.

MATERIALS AND METHODS

Reagents, antibodies, and cell lines

All cell culture reagents were purchased from Gibco/BRL Life Technologies Ltd. (Paisley, Scotland). Plates,

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Abbreviations: PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; IL-1, Il-2, Il-6, interleukins 1, 2, and 6, respectively; TNFα, tumor necrosis factor α; FCS, fetal calf serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline solution; TBS, tris-buffered saline solution; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol-12-myristate 13-acetate; PKC, protein kinase C.

flasks, and bottles were from Costar (Cambridge, MA, U.S.A.). Polyacrylamide gel electrophoresis equipment and reagents were from Bio-Rad (Richmond, CA, USA). Horseradish peroxidase-linked secondary antibodies and enhanced chemiluminesence (ECL) reagents were obtained from Amersham (Amersham, U.K.). Protein A-sepharose was from Pharmacia (Pharmacia Biotech. Europe, Brussels, Belgium). Staurosporine and bisindoyl maleimide were from Boehringer Mannheim (Mannheim, Germany). Mouse monoclonal anti-EGF receptor antibody (05-104) and rabbit polyclonal anti-phosphotyrosine (06-123) were from Upstate Biotechnology Inc. (UBI, Lake Placid, NY, U.S.A.). PY20 anti-phosphotyrosine monoclonal antibody was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA, U.S.A.). Anti-human cMet β-chain C28 terminal peptide rabbit polyclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.), and is subsequently referred to in the text as 'polyclonal anti intracellular cMet β'. Purified DL-21 (a mouse monoclonal used for Western blotting recognising a human cMet β-chain extracellular epitope), DO-24 (a mouse monoclonal used for immunoprecipitation that recognises a native extracellular epitope of the human cMet β-chain), DQ-13 (a mouse monoclonal anti-human cMet raised against a 19 amino acid peptide corresponding to the carboxy-terminus of the cMet β-chain) ascites fluid, and the GTL-16 (human gastric tumour) cell line were generously provided by Paolo Comoglio, University of Turin, Italy. A549 (ATCC CCL 185) and A431 (ATCC CRL 1555) cell lines were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Suramin (Bayer 205) was from Bayer (Leverkusen, Germany). All other reagents, unless otherwise stated, were from the Sigma Chemical Company (St. Louis, MO, U.S.A.).

Treatment of cells

GTL-16 cells were seeded at a density of 2×10^5 cells/cm^2 in 6-well plates in RPMI supplemented with 10% fetal calf serum (FCS) and grown to near confluence, then incubated overnight in RPMI starvation medium (RPMI supplemented with 0.5% FCS and 0.1% BSA). The cells were then washed twice with PBS and once with starvation medium, then incubated for the indicated times at 37°C (unless otherwise stated) in 2 ml of starvation medium containing the stated concentration of drug.

A549 cells were seeded at a density of 5×10^4 cells/cm^2 in 6-well plates and incubated in DMEM containing 10% FCS. When confluent, they were incubated overnight in starvation medium (DMEM supplemented with 0.5% FCS and 0.1% BSA), and treated as described above for GTL-16.

A431 cells were seeded at a density of 1×10^5 cells/cm^2 in 6-well plates and incubated overnight in high-glucose DMEM (4.5 g/l glucose) containing 10% FCS. The cells were grown to confluence, then incubated overnight in starvation medium (high-glucose DMEM supplemented with 0.5% FCS and 0.1% BSA), and treated as described for GTL-16.

Surface labelling with biotin

GTL-16 cells grown in 6-well plates and treated with suramin as described above were surface-labelled with biotin using the Cellular Labelling Kit produced by Boehringer Mannheim (Mannheim, Germany). Labelling was performed according to the manufacturer's instructions in a reaction volume of 500 µl/well. Each well contained 2×10^6 cells.

Western blots of cell lysates and supernatants

After treatment of cells, culture supernatants were collected and centrifuged at 4°C, first at 1000 × g for 10 minutes, and then at 15000 × g for 30 minutes. The cells were washed twice with cold PBS, then lysed by addition of 0.5 ml of cold lysis buffer (150 mM NaCl, 50 mM HEPES/NaOH, pH 7.4, 5 mM EDTA, 1% Triton X-100, 1% Na deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 30 mM para-nitrophenyl phosphate, 1 mM sodium orthovanadate, 0.1 mM ammonium molybdate, 30 mM sodium fluoride, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 20 µg/ml aprotinin, and 20 µg/ml leupeptin). After cell lysis, the lysates were cleared by centrifugation for 15 minutes at 15000 × g. Protein concentration determinations were performed on the cell lysates using the bicinchoninic acid assay (BCA, Pierce, Rockford, IL, U.S.A.) with bovine serum albumin as standard. The indicated amounts of protein were prepared for electrophoresis in reducing Laemmli buffer, and run on SDS-polyacrylamide gels (7.5% polyacrylamide) using Bio-Rad Broad Range molecular weight markers (Bio-Rad, Richmond, CA, U.S.A.) as standards. After electrophoresis, proteins were transferred onto nitrocellulose filters for 90 minutes at a constant current of 300 mA in buffer consisting of 192 mM glycine, 25 mM Tris, and 20% (v/v) methanol, pH 8.3. Filters were incubated overnight at 4°C in Tris-buffered saline (TBS, 20 mM Tris, 140 mM NaCl, 0.1% Tween 20, pH 7.6) containing 5% BSA (TBS-BSA) to block nonspecific sites. Blocked filters were probed for two hours with TBS-BSA containing 1 µg/ml of primary antibody (unless otherwise stated), then washed in TBS, probed for 90 minutes with a 1/4000 dilution of horseradish peroxidase-labelled secondary reagent in TBS/BSA (anti-mouse Ig, anti-rabbit Ig, or streptavidin, as appropriate, all from Amersham), then washed and developed using an enhanced chemiluminesence system (Amersham). Blots were stripped by washing for 30 minutes in 0.1 M dithiothreitol, 2% SDS, 50 mM Tris, pH 7.5, and then 30 minutes at 50°C in 50 mM Tris, pH 7.5, containing 2% SDS. Stripped filters were washed twice for 10 minutes in TBS, then blocked and reprobed with the stated antibody, as described above.

Immunoprecipitation of cMet

cMet was immunoprecipitated from cleared lysates or conditioned media by addition of (as appropriate) 1 µl DQ-13 ascites, or 0.2 µg of DO-24 Ig and 50 µl (packed volume) protein A-sepharose per 0.5 ml of reaction. Reactions were incubated with shaking at 4°C for 3 hours, after which the protein A-sepharose pellets were washed twice in 0.5 ml lysis buffer, resuspended by addition of 2X reducing Laemmli loading buffer, and subjected to SDS-PAGE and Western blotting as described above.

Densitometric analysis of Western blots

This was performed using a Pharmacia LKB Ultroscan XL Laser Densitometer (Pharmacia Biotech. Europe, Brussels, Belgium) according to the manufacturer's instructions.

RESULTS

Suramin treatment of GTL-16 cells reduces cell-associated cMet

The GTL-16 cell line is derived from a human gastric carcinoma [19], and overexpresses a constitutively activated but unmutated form of cMet [20]. Figure 1 shows the results of a typical experiment in which confluent cultures of GTL-16 were incubated with 300 μM suramin for 6 hours at 37°C. We have previously found that this dose of suramin is effective in neutralising HGF-induced scatter activity, but has no acute toxicity on most cell lines tested, including GTL-16 [21]. After treatment, culture media were collected, and the intact cells surface-labelled with biotin [22]. Cells were then lysed, and cell lysates and conditioned culture media analysed by SDS-polyacrylamide gel electrophoresis followed by Western blotting using either streptavidin as a probe for cell surface (i.e. biotin-labelled) proteins, or anti-cMet specific antibodies. Under these conditions, suramin does not cause generalised protein degradation in GTL-16 cells, since no obvious differences in the SDS-PAGE band pattern can be discerned when untreated and suramin-treated cell lysates are compared after Coomassie staining (Fig. 1A). However, when lysates of cells are Western-blotted using streptavidin as a probe for cell-surface proteins, a clearly detectable difference between untreated and suramin-treated cells is that of a decrease in a single band of approximately 140 kDa (Fig. 1B). Western blotting of cell lysates and conditioned culture media using the monoclonal antibody DL-21 (Fig. 1C), which recognises an epitope on the extracellular portion of the cMet β-chain [14], demonstrates that incubation with suramin results in decreased levels of full-length cell-associated cMet β-chain. This species has the same electrophoretic mobility as the cell-surface biotin labelled band, which specifically decreases in intensity upon suramin treatment. The decrease in cell-associated full-length cMet β is accompanied by a concomitant increase in the culture medium of a DL-21 immunoreactive band (cMet β′) with an apparent molecular weight, when reduced, of approximately 83 kDa. Although this is slightly larger than the 75 kDa previously observed soluble form of cMet β [14], this species (i.e. cMet β′) comigrates both with the constitutively secreted form of cMet β and that induced by phorbol ester (see below Figs. 3 and 8), implying that discrepancies in estimated sizes of soluble cMet β are due to differences in electrophoresis conditions and molecular weight standards employed. When run non-reduced (data not shown), the apparent molecular weight of cMet β′ shifts upwards by approximately 50 kDa, consistent with the previous observation [14] that the shed form of the HGF receptor consists of most of the extracellular portion of the cMet β-chain covalently bound via disulphide linkage to the α-chain.

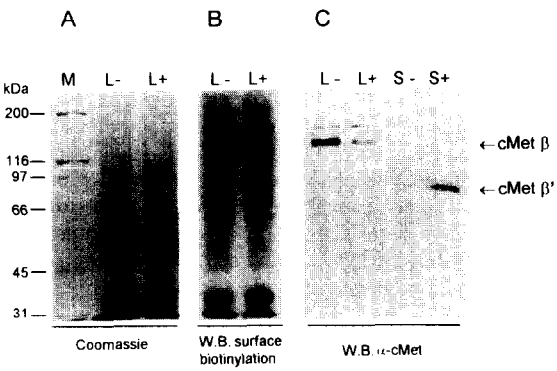

Fig. 1. Suramin induces selective shedding of cell surface cMet β. GTL-16 cells were incubated in the absence or presence of 300 μM suramin for 6 hours; cell culture supernatants were collected, and surface proteins on intact cells labelled with biotin, then cells were lysed as described in Materials and Methods. Eight μg of cell lysate proteins or a volume of cell culture supernatant (i.e. conditioned culture medium) corresponding to the same quantity of cells was electrophoresed and Western blotted. (A) Coomassie-stained gel of cell lysates. M, molecular weight standards. L–, L+ indicate lysates of cells incubated in absence or presence, respectively, of suramin. (B) Western blot (W.B.) of the cell lysates probed with a 1/4000 dilution of streptavidin/horseradish peroxidase conjugate. L–, L+ as in previous panel. (C) Western blot of cell lysates and culture supernatants probed with DL-21, a monoclonal antibody specific for an extracellular epitope of cMet β. L–, L+ indicate lysates of cells incubated in absence or presence of suramin. S–, S+ indicate the corresponding culture supernatants.

Induction of soluble cMet shedding by suramin is dose- and time-dependent

The effect of suramin in receptor shedding is dose-dependent; after 6 hours' incubation, an increase in the culture medium of cMet β′ (Fig. 2A) and an accompanying decrease of the cell-bound (full-length) form of cMet β (Fig. 2B) are discernible at 100 μM suramin, and this phenomenon continues to become more pronounced up to 1 mM. Note that suramin induced loss of full-length cMet β is accompanied by a dramatic decrease in tyrosine phosphorylation of cellular proteins (Fig. 2C), probably due to loss of the tyrosine kinase activity of cMet consequent to its cleavage.

When confluent layers of GTL-16 are incubated for various times with 300 μM suramin, and the conditioned media analysed by Western blotting using the antibody DL-21 as a probe (Fig. 3), increased levels of cMet β′ are detectable after approximately 10 minutes, indicating that induction of soluble cMet by suramin is a rapid process. A relative evaluation of the degree to which the full-length receptor is degraded was effected by performing gel electrophoresis/Western blotting on increasing amounts (from 0 to 10 μg) of lysate protein from untreated cells to produce a standard curve, loaded alongside 10 and 5 μg of lysate protein from cells treated with suramin. Anti-cMet Western blots of such gels were analysed by laser densitometry (data not shown), and the residual amount of whole cMet β remaining in the lysates of suramin-treated cells was calculated by comparing the cMet band density of the suramin-treated sample with the internal standard curve. This analysis, performed three times on separately treated cells, showed that a 6-hour exposure of GTL-16 cells to 300 μM suramin reduced the amount of cell-associated cMet to 48% (standard deviation = 15) of original levels (data not shown).

cMet shedding results in the appearance of a complementary intracellular fragment (cMet β″)

Extracellular proteolytic cleavage of cMet leading to formation of a soluble receptor should result in the concomitant production of a complementary intracellular C-terminal fragment of the cMet β chain containing the

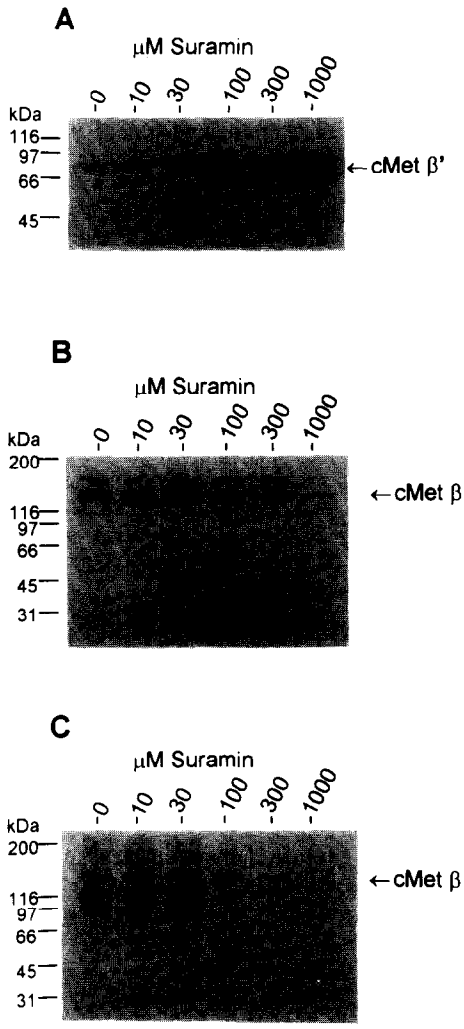

Fig. 2. Dose-response of cMet shedding induced by suramin in GTL-16 cells. GTL-16 cells were incubated for 6 hours at 37°C with the indicated concentrations of suramin, then supernatants (culture media) were collected and cells lysed as described in Materials and Methods. Eight μg lysate proteins and supernatants corresponding to an equivalent number of cells were electrophoresed and Western blotted. (A) Supernatants and (B) cell lysates probed with monoclonal antibody DL-21. (C) Same filter as in (B), stripped and re-probed with PY20 anti-phosphotyrosine monoclonal.

kinase domain. However, in experiments such as those described above, we could obtain no evidence of this fragment when lysates of GTL-16 cells treated with suramin were Western blotted and probed with polyclonal anti-intracellular cMet β antibodies (not shown). The expected intracellular fragment was also undetectable in a previous study in which phorbol-12-myristate 13-acetate (PMA) was used to induce formation of soluble cMet [14]. In that case, failure to detect it was ascribed to its possible rapid degradation. To test this hypothesis, GTL-16 cells were incubated with suramin at 4°C and 37°C, since slowing down cellular metabolism might allow the detection of C-terminal fragments of cMet β, which would otherwise become rapidly degraded. This is indeed the case: Western blotting of cell lysates and conditioned media with monoclonal antibody

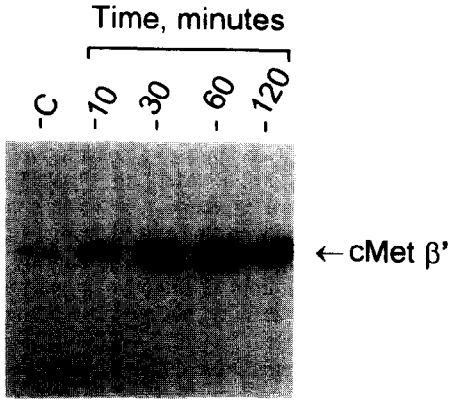

Fig. 3. Time-course of induction of cMet β′ in conditioned media of GTL-16 cells treated with suramin. Confluent layers of GTL-16 were incubated for the indicated times with 300 μM suramin, as described in Materials and Methods. After incubation, conditioned media were collected, and 30 μl aliquots were analysed by Western blotting using monoclonal antibody DL-21 as a probe. C indicates the conditioned medium from cells incubated in absence of suramin for 120 minutes.

DL-21 showed that suramin-induced shedding of cMet β′ is able to proceed both at 4°C and 37°C, (Fig. 4A), whereas blotting of cell lysates with polyclonal anti-intracellular cMet β antibodies revealed the presence of a doublet band (cMet β″) of approximately 60 kDa molecular weight in cells treated with suramin at 4°C, but not at 37°C (Fig. 4B). Moreover, probing with an anti-phosphotyrosine monoclonal antibody (PY20) showed that this band is phosphorylated on tyrosine residues, as would be expected for a fragment containing the kinase domain of cMet (Fig. 4C).

The identity of this doublet as a C-terminal fragment(s) of the cMet β chain was confirmed by immunoprecipitation experiments using monoclonal antibodies specific for either the intracellular or extracellular portions of cMet β; cMet β″ detection in Western blots of immunoprecipitates is possible if cell lysates are immunoprecipitated using DQ-13, a monoclonal specific for an intracellular epitope of cMet β (Fig. 5A), but not if DO-24, a monoclonal specific for a cMet β extracellular epitope, is used (Fig. 5B—note that the band of approximately 55 kDa running across all lanes is due to cross reaction between the secondary antibody used to develop the blot and the heavy chain of antibody DO-24; cMet β″ has a lower mobility, and would not be masked by this band). Figure 5C confirms that the immunoprecipitated cMet β″ is phosphorylated on tyrosine. The sum of the apparent molecular weights of the cMet β″ fragment and of the reduced shed portion of the receptor (cMet β′), approximately 60 and 83 kDa, respectively, are in good agreement with the observed molecular weight of the integral β-chain (145 kDa). The disappearance of cMet β″ in cells treated at 37°C implies that degradation of this species is normally extremely rapid.

Suramin induces cMet shedding in cell lines not overexpressing the receptor

To verify whether suramin-induced cMet shedding is restricted to GTL-16 (a line that might constitute an unrepresentative model for the study of cMet shedding, since these cells overexpress a constitutively active form of the receptor), A549 [23] and A431 [24], two human

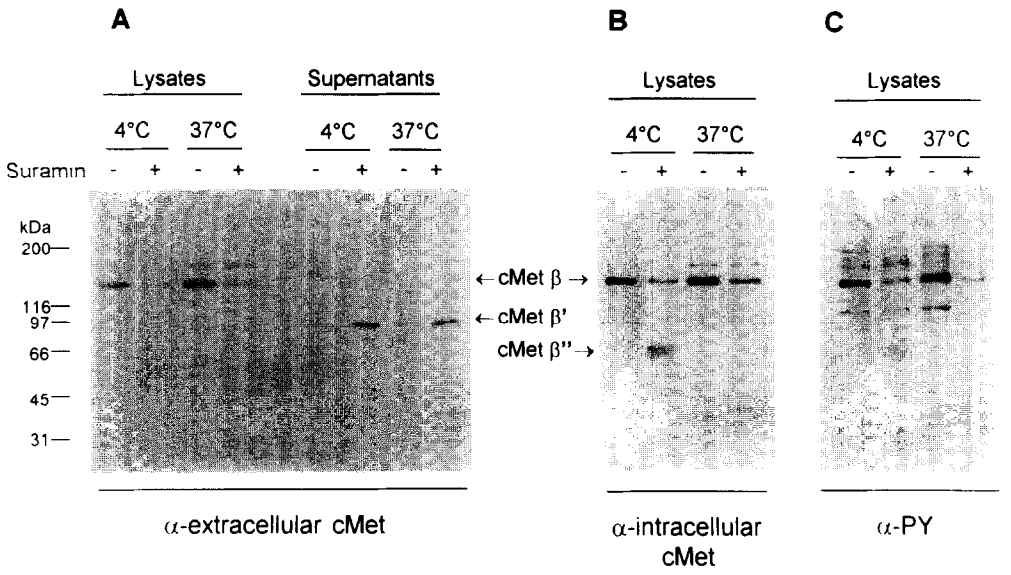

Fig. 4. Treatment of GTL-16 at 4°C and 37°C with suramin. GTL-16 cells were incubated for 6 hours with or without 300 μM suramin at the indicated temperatures, then culture supernatants were collected and cells lysed, as described in Materials and Methods. Eight μg of cell lysate proteins, or a corresponding volume of supernatant were electrophoresed and Western blotted. (A) Lysates and supernatants probed with DL-21. (B) Lysates probed with polyclonal anti-intracellular cMet β. (C) Lysates probed with PY20 anti-phosphotyrosine (α-PY).

carcinoma-derived cell lines that normo-express non-constitutively activated cMet, were treated with suramin under the same conditions as those used for GTL-16 cells. Downregulation of cell-associated full-length cMet and upregulation of secreted soluble cMet were observed in A549, a pulmonary carcinoma-derived cell line (Fig. 6). Suramin treatment had no effect on levels of the epidermal growth factor receptor in A431 cells, which overexpress a constitutively activated form of this receptor tyrosine kinase (Fig. 7A), but was still able to induce cMet shedding (Fig. 7B).

Suramin causes shedding of cMet by a mechanism independent of protein kinase C

Phorbol esters such as PMA specifically activate protein kinase C (PKC) by binding to the enzyme's regulatory domain [25]. To determine whether cMet shedding induced by suramin is dependent on PKC activity, GTL-16 cells were treated with either PMA or suramin in the presence of staurosporine, a potent inhibitor of several protein kinases including PKC, and bisindoyl maleimide, a much more specific inhibitor of protein kinase C [26]. Both these compounds strongly inhibit the increased cMet shedding induced by PMA, whereas that induced by suramin proceeds unaffected (Fig. 8). These data indicate that suramin and PMA activate the processes that lead to cMet shedding via distinct mechanisms, even though the cMet β′ species produced by both agents have apparently identical electrophoretic mobilities.

DISCUSSION

Treatment of cells expressing cMet with suramin results in the rapid secretion of a soluble form of the receptor, which seems to consist of a C-terminal truncated β-chain, cMet β′, and of the intact α-chain of cMet. The truncated form of cMet β induced by suramin has an apparently identical electrophoretic mobility to that induced by PMA and to the constitutively secreted species, raising the possibility that both of these agents act by upregulating the rate of an endogenous pathway for the formation and secretion of the soluble receptor.

It has previously been found that induction of soluble cMet by PMA is due to proteolytic processing of the integral receptor at the extracellular surface of the cell membrane rather than the production of an alternatively spliced mRNA encoding a truncated receptor, since appropriate transcripts are undetectable in Northern blots of GTL-16 cells, and since transfection of mouse cells with cDNA encoding full-length human cMet results in production of soluble as well as the regular cell-bound human receptor [14]. The reciprocal relationship between decreased levels of cell-associated full-length cMet β and the appearance of cMet β′ in supernatants of cells treated with suramin suggest that this agent also induces proteolytic generation of soluble cMet. Moreover, we demonstrate for the first time the presence of the complementary intracellular fragment of the truncated receptor, cMet β″, and its increased production in cells induced to shed cMet with suramin. Since this fragment becomes detectable only when cellular metabolism is decreased by treatment at low temperatures, a plausible explanation for previous failures to detect it may be that extremely rapid internalisation and/or degradation of the membrane-bound C-terminal fragment occurs as a consequence of shedding. This is to be expected, since one presumes that a receptor tyrosine kinase lacking the extracellular domain is potentially deregulated, and mechanisms for its rapid removal must exist to prevent deleterious consequences to the cell. The ability of suramin to induce soluble cMet secretion efficiently at low temperatures is surprising, particularly since at least one report has found that TNFα receptor shedding is inhibited at temperatures below 16°C [27], but perhaps provides evidence that processes involving a high degree

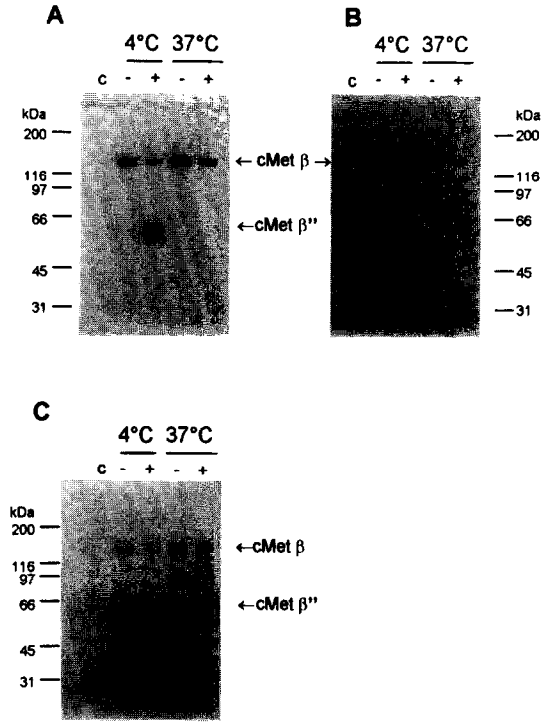

Fig. 5. Immunoprecipitation of the intracellular fragment of cMet β (cMet β″) containing the tyrosine kinase domain. Cell lysates from the experiment depicted in Fig. 4 were immunoprecipitated with either (panels A and C) DQ-13 (a monoclonal antibody specific for a cMet β intracellular antigen), or (panel B) DO-24 (a monoclonal specific for a cMet β extracellular antigen), then electrophoresed and Western blotted as described in Materials and Methods. Immunoprecipitates from 10 μg of cellular proteins were loaded in each lane. Lanes marked c correspond to antibody immunoprecipitated in absence of cell lysate, lanes marked – correspond to immunoprecipitates from cells incubated in absence of suramin, and + from cells incubated with suramin. Panels (A) and (B) were probed with polyclonal anti-intracellular cMet β antibody and panel (C) with 10 μg/ml of polyclonal anti-phosphotyrosine.

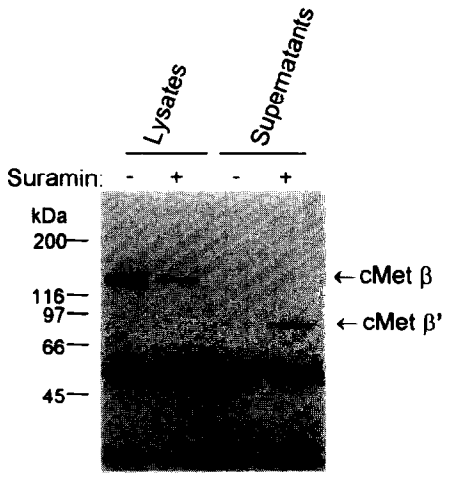

Fig. 6. Suramin-induced cMet shedding in A549 cells. Cells were incubated with or without 300 μM suramin for 6 hours, then supernatants collected and cells lysed. Lysates and supernatants were immunoprecipitated with monoclonal antibody DO-24. Immunoprecipitates from 100 μg of cellular proteins, or a volume of culture supernatant corresponding to an equivalent number of cells were electrophoresed and Western blotted as described in Materials and Methods. The filter was probed with antibody DL-21 (anti-extracellular cMet β).

of cellular metabolism (e.g. membrane internalisation) are not requisite for cMet shedding.

The mechanisms by which PMA and suramin activate cMet shedding are at least partially distinct, since PMA exerts its effect via PKC, whereas suramin does not, as shown by use of PKC inhibitors. We have observed a further dissociation of the shedding activated by suramin and PMA (data not shown), in that whereas overnight treatment of GTL-16 cells with PMA leads to a full recovery of cMet levels and renders the cells refractory to further PMA-induced shedding, downmodulation of cMet by suramin persists for as long as the agent is present, but is reversible upon its removal. Although the significance of soluble cMet induction by agents such as PMA and suramin is as yet unclear, it is perhaps worth noting that activation of PKC by phorbol esters leads to inactivation of the tyrosine kinase activity of the receptor by phosphorylation of a regulatory residue, serine 985, residing within the intracellular juxtamembrane region of cMet β [28]. It is tempting to speculate that this activity of PKC and its ability to generate shedding of the extracellular domain of cMet are related, since both represent a means for negatively modulating receptor activity.

The putative 'shedding protease' seems extremely unusual, since we were unable to inhibit cMet β′ induction using a wide range of protease inhibitors including aprotinin, α_1-antitrypsin, antithrombin III, leupeptin, phenylmethylsulfonyl fluoride (PMSF), Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), phosphoramidon, trans-epoxysuccinyl-L-leucylamido (4-guanidino)butane (E-64), pepstatin, and o-phenanthroline (data not shown). With one exception [29], failure to inhibit shedding with common protease inhibitors has also been the experience of several other workers investigating different shed receptors [15], perhaps implying that shedding proteases belong to a so far uncharacterised group of proteolytic enzymes.

Previous studies (reviewed in ref. [2]) have shown that suramin is able to inhibit the biological activity of many growth factors, particularly those that are heparin-binding (which include HGF), by associating with the factor, perhaps via interactions similar to those of heparin, resulting in an abrogation of receptor binding. However, suramin is able to inhibit an array of different growth factors and cytokines, many of which are not heparin-binding, and although several studies have demonstrated its capacity to displace from receptors growth factors that bind with exceedingly low dissociation constants, such as PDGF, bFGF, and vascular endothelial growth factor [3, 4, 30], a detailed analysis of the direct binding of suramin to these growth factors is still lacking a decade after the first such observations [2]. In our view, it is therefore interesting to note that apart from cMet, proteolytic shedding has been described for the receptors of several other growth factors inhibited by suramin, amongst which are those for Il-1 [31], Il-2 [32], Il-6 [17], nerve growth factor, [33], PDGF [34], transferrin [35], and TNFα [18]. Also intriguing is a recent finding that suramin is able to cause a reduction in cell-surface levels of CD4 as measured by cell-binding studies with anti-

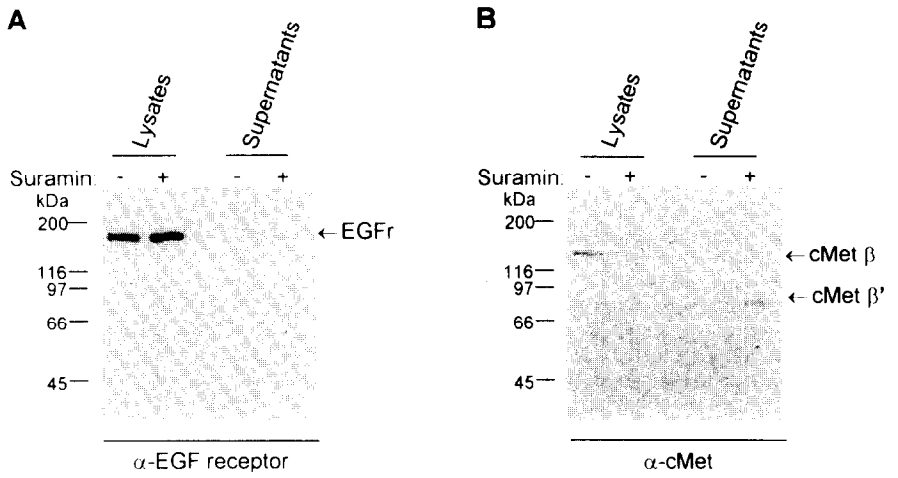

Fig. 7. Suramin-induced cMet shedding in A431 cells. Cells were incubated with or without 300 μM suramin for 6 hours, then supernatants collected and cells lysed. Twenty μg of cell proteins, or a corresponding volume of cell culture supernatant was electrophoresed and Western blotted. In panel (A), the filter was probed with 1 μg/ml of a monoclonal antibody specific for the extracellular domain of the epidermal growth factor receptor (EGFr). Panel (B): the same filter stripped and re-probed with 1 μg/ml DL-21 (anti-extracellular cMet β).

CD4 antibodies [36]. CD4, a helper T-lymphocyte antigen responsible for binding of HIV to human lymphocytes, was found to be lost from the surface of human peripheral blood lymphocytes *in vitro*, and from cultured cell lines at incubation times and doses of suramin consistent with our findings regarding loss of cell surface cMet; however, the question of whether this modulation might proceed via proteolytic shedding was not addressed.

In conclusion, the plethora of biological activities so far ascribed to suramin and the wide array of different factors that it is able to inhibit make it unlikely that this agent intervenes in growth factor interactions with receptors and subsequent receptor activity via a single mechanism such as binding to the factor and prevention of receptor binding. Indeed, we have previously shown that suramin and suramin analogues are able to inhibit HGF-induced activation of cMet at doses and times at which shedding is undetectable [21], so that inhibition of this growth factor possibly involves two different mechanisms. Whether the previously undescribed ability of suramin to stimulate receptor shedding is peculiar to cMet, or can be extended to other receptors, is potentially of great interest in the search for agents capable of modulating receptor activity, and a subject we are currently attempting to investigate.

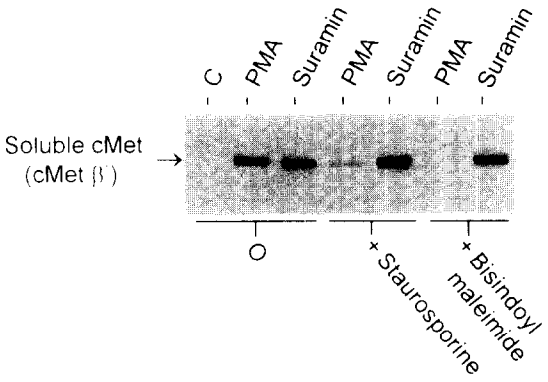

Fig. 8. PMA and suramin-induced cMet shedding in GTL-16 cells, and effects of PKC inhibitors. GTL-16 cells were incubated for 30 minutes with medium containing no additive (C), 100 ng/ml PMA, or 300 μM suramin, or with the same concentrations of PMA or suramin in the presence of 5 μM staurosporine or bisindoyl maleimide; 20 μl aliquots of each cell culture supernatant were electrophoresed and Western blotted as described in Materials and Methods. The filter was probed with DL-21 (anti-extracellular cMet β).

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