Role of the Cys^{90}, Cys^{95} and Cys^{173} residues in the structure and function of the human platelet-activating factor receptor

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Received 3 June 1996; revised version received 20 December 1996

Abstract Platelet-activating factor (PAF) is a potent phospholipid mediator which binds to a specific, high affinity receptor of the G protein-coupled receptor family. In the present report, we show that ligand binding to the PAF receptor is sensitive to the reducing agent dithiothreitol (DTT), suggesting the involvement of disulfide linkages in the proper PAF receptor conformation. Substitutions of Cys^{90}, Cys^{95} and Cys^{173} to Ala or Ser demonstrated that these cysteine residues are critical for normal cell surface expression of the PAF receptor protein and ligand binding to the receptor. The Cys^{90} and Cys^{173} mutant receptors did not display any specific ligand binding, were not expressed on the cell surface but were found in the intracellular compartment. The Cys^{95} mutants showed specific binding and were able to stimulate low levels of inositol phosphate (IP) production. These mutants were expressed at low density on the cell surface and showed high expression intracellularly. Our results suggest that the structure and function of the PAF receptor require the conserved Cys^{90} and Cys^{173} to form a disulfide bond. Moreover, Cys^{95} also appears to be necessary, possibly by establishing a disulfide linkage with an as yet unidentified Cys residue. All three residues appear essential for the proper folding and surface expression of the PAF receptor protein.

Key words: G protein-coupled receptor; PAF receptor; Disulfide bond

1. Introduction

Seven transmembrane-spanning, guanine nucleotide-binding (G) protein-coupled receptors (GPCRs) constitute a large family of cell surface regulatory molecules. Because of the evidence that has accumulated to implicate disulfide and thiol groups in ligand binding and agonist activation, cysteine residues have been the subject of considerable investigation using mutagenesis and biochemical techniques. The majority of GPCRs sequenced to date contain a pair of conserved Cys residues in the second and third extracellular domains. It has been assumed that this pair of Cys residues forms a disulfide bond in most GPCRs. This linkage has been proposed to be important to allow the receptor to attain a normal conformation during synthesis, for proper cell surface expression and function, in particular, binding and activation [1]. For some receptors of the GPCR family, data consistent with the presence of a disulfide bond between these two conserved Cys residues have been reported, but there is evidence that these conserved residues do not always participate in a disulfide linkage. For example, data from a series of β_2-adrenergic mutants show that there is no disulfide bond between the two conserved Cys [2].

The PAF receptor is a member of the GPCR superfamily [3–7]. The human PAF receptor amino acid sequence contains twelve Cys residues. The two residues are found in the second (Cys^{90}) and the third (Cys^{173}) extracellular domains were proposed to form disulfide bond, based on a molecular model of the receptor [8]. Another Cys residue (Cys^{95}), found at the border of the second extracellular domain and the third transmembrane domain, could potentially be involved in the formation of the disulfide linkage with Cys^{173}, instead of Cys^{90}. Therefore, site-directed mutagenesis and transient expression of PAF receptor mutants with Cys amino acid substitutions were used to identify the role of these three residues in the structure and function of the receptor and to verify which of the Cys^{90} or Cys^{95} residues was forming a disulfide bond with Cys^{173}.

2. Materials and methods

2.1. Construction of the mutant receptor cDNAs and expression vectors

Mutated receptors were constructed by polymerase chain reaction (PCR) [9] using the PAF receptor cDNA from Kp132 as template (a generous gift from Dr. Richard Ye, The Scripps Research Institute, La Jolla, CA) [6]. Mutant oligonucleotides were as follows: C90A, 5′-CCAAATTCCTGGCCAACGTGGC-3′; C90S, 5′-CCAAATTCCT-GTCCAACGTGGC-3′; C95A, 5′-GTGGCTCCCGCCCTTTTC-T-TC-3′; C95S, 5′-TGGCTGGCAGCCTTTTC-3′; C173A, 5′-GTCA-CTCGCGCCTTTGAGCATTAC-3′; C173S, 5′-CGTCA-CTCGCTC-CTTTGAGC-3′ and their respective reverse complements. The mutant PCR products were digested with *Bgl*II-*Bst*EII and subcloned into the pJ3M vector (kindly provided by Dr. J. Chernoff, Fox Chase Cancer Center, Philadelphia, PA) [10] containing the WT receptor, in frame with the 'cmyc' epitope, also digested with *Bgl*II-*Bst*EII. The region corresponding to the subcloned PCR fragments was sequenced on both strands by dideoxy sequencing of double-stranded DNA with Sequenase (U.S. Biochemical Corp.).

2.2. Cell culture and transfections

COS-7 and CHO cells were grown in Dulbecco's modified Eagle's medium (high glucose) and Dulbecco's modified Eagle's medium F-12 (Ham's medium, high glucose), respectively, supplemented with 10% fetal bovine serum. A stable line of CHO cells expressing WT PAF receptor [11] were cultivated in the presence of 400 μg/ml of G418. Cells were plated in 30-mm dishes (2×10^5 cells/dish), transiently transfected with the constructions encoding the WT and the mutant receptors using 4 μl of lipofectamine (Life Technologies, Inc.) and 1 μg of DNA per dish and harvested 48 h after transfection.

2.3. Radioligand binding assay

Competition binding studies were done on CHO cells expressing the wild-type and mutant receptor species. Cells were harvested and washed twice in HEPES-Tyrode's buffer (140 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.6 mM D-glucose, 0.49 mM $MgCl_2$, 0.37 mM NaH_2PO_4, 25 mM HEPES pH 7.4) containing 0.1% (w/v) bovine serum albumin [12]. Binding reactions were carried out on 5×10^4 cells in a total volume of 0.25 ml in the same buffer with 10 nM [^{3}H]WEB2086 (Du Pont-New England Nuclear) and increasing concentrations of nonradioactive WEB2086 for 90 min at 25°C. Reactions were stopped by centrifugation. The cell-associated radioac-

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PII S0014-5793(96)01531-1

tivity was measured by liquid scintillation. Membrane fractions were prepared and binding carried out as previously described [11]. Binding reactions involving [^{3}H]PAF (4 nM) were performed on intact adherent CHO cells in a total volume of 600 μl of binding buffer containing 150 mM choline chloride, 10 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5, and 0.25% lipid-free bovine serum albumin [13]. Samples were incubated for 4 h at 4°C. Cells were washed twice with ice-cold buffer (1 ml), lysed with 0.1 N NaOH, and analyzed for radioactivity [13]. In selective experiments, cells were preincubated with DTT (10 mM) [1] ± selenol (0.8 mM) (selenol was generated in situ from selenocystamine, Sigma) [14] for 60 min at room temperature prior to performing binding reactions. DTT ± selenol was present at the same concentrations during the binding reactions.

2.4. Inositol phosphate determination

COS-7 cells were transfected as described above with the wild-type or mutant receptors and labeled the following day for 18–24 h with [^{3}H]*myo*-inositol (Amersham) at 5 μCi/ml in Dulbecco's modified Eagle's medium (high glucose, without inositol) (Life Technologies, Inc.). After labeling, cells were washed once in phosphate-buffered saline (PBS) and preincubated 5 min in PBS at 37°C. At the end of this preincubation period, the PBS was removed and cells were incubated in pre-warmed Dulbecco's modified Eagle's medium (high glucose, without inositol) containing 0.1% (w/v) bovine serum albumin and 20 mM of LiCl, for 5 min. Cells were then stimulated for 30 s with indicated concentrations of PAF. The reactions were terminated with the addition of perchloric acid followed by a 30 min incubation on ice. Inositol phosphates (IPs) were extracted [15] and separated on Dowex AG1-X8 (Bio-Rad) columns [16]. Total labeled IPs were then counted by liquid scintillation.

2.5. Flow cytometry studies

CHO cells transfected with cmyc-tagged mutant and WT receptor constructions were harvested 48 h after transfection and subjected to flow cytometry analysis. Cells (2.5×10^5) were washed twice in PBS and labeled with anti-cmyc antibody (9E10 hybridoma, ATCC) or with an isotype control antibody (anti-HA, epitope 12CA5 of influenza hemagglutinin protein, Boehringer Mannheim) at room temperature (RT) for 30 min. Cells were then washed with PBS and incubated at RT for an additional 30 min with fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Bio/Can). All measures were performed on a FACScan flow cytometer (Becton-Dickinson).

2.6. Confocal microscopy

Transiently transfected CHO cells were grown on coverslips (25 mm). 48 h after transfection the cells were fixed with 4% paraformaldehyde (15 min at RT). The coverslips were then placed in 0.1% Triton (20 min RT), then sequentially incubated with 5% milk (30 min RT) and 0.1% glycine (60 min RT). The cells were then incubated with anti-cmyc or isotype control antibodies as described for flow cytometry studies. The cells were then analyzed on a Molecular Dynamics (Sunnyvale, CA) Multi Probe 2001 confocal argon laser scanning system equipped with a Nikon Diaphot epifluorescence inverted microscope. Scanned images were transferred onto a Silicon Graphics Indy 4000 workstation equipped with Molecular Dynamics' Imagespace analysis software.

3. Results and discussion

In order to study the role of Cys^{90}, Cys^{95} and Cys^{173} residues in the structure and function of the human PAF receptor, these Cys were individually altered to Ser and Ala using site-directed mutagenesis. The mutant receptors were transiently expressed in COS-7 and CHO cells for evaluation of binding pharmacology and cell signaling. Fig. 1 illustrates the position of the mutated Cys relative to the overall molecular structure of the PAF receptor.

3.1. Effects of disulfide bond reduction on ligand binding

Fig. 2A shows the effect of the reducing agent DTT on specific [^{3}H]WEB2086 and [^{3}H]PAF binding. Selenol has been reported to increase the rate of disulfide bond reduction

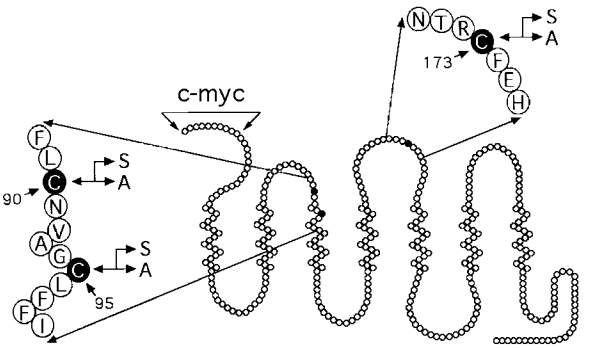

Fig. 1. Putative seven-transmembrane-segment topography of the PAF receptor. Solid circles indicate the amino acids of the PAF receptor that were mutated and the substituting residues are illustrated.

by DTT [14]. In presence of DTT alone or with selenol, specific [^{3}H]PAF binding to the WT receptor was only ≈60% and ≈63% of that to the untreated receptors, respectively. In addition, similar effects were observed for the antagonist [^{3}H]WEB2086 with ≈46% (DTT) and ≈42% (DTT+selenol) residual binding. These results suggest that disulfide bonds are required for proper binding of structurally distinct ligands of the PAF receptor. In these experiments, the addition of selenol to the reaction did not potentiate the effect of DTT. In Fig. 2B,C, binding isotherms revealed no change in receptor affinity for [^{3}H]WEB2086 or [^{3}H]PAF when incubated with or without DTT ± selenol. This indicates that the binding sites observed after treatment were probably intact receptors.

3.2. Analysis of the mutant receptor ligand binding and cell signaling

Fig. 3A indicates the specific [^{3}H]WEB2086 and [^{3}H]PAF binding to the mutant and the WT receptors. Only the C95A and the C95S mutants displayed detectable specific binding, although too weak to be able to produce reliable equilibrium binding isotherms. The C90A, C90S, C173A and C173S mutant-expressing cells did not show any specific binding for either ligand. Binding reactions carried out on crude membrane preparations gave the same results (data not shown), suggesting that these mutations produce receptors incapable of binding their ligands. In order to determine whether the substitution mutant PAF receptors were appropriately coupled to signaling effector molecules, we measured the production of IPs in transiently transfected COS-7 cells following a 30-s stimulation over a $0–10^{-6}$ M PAF concentration range. PAF concentrations higher than 10^{-6} M were not used as PAF has been shown to have non-receptor mediated effects at these concentrations [17]. Fig. 3B demonstrates that the WT receptor attained a maximal response at ≈10^{-7} M PAF and a half-maximal response at ≈10^{-8} M PAF (IC_{50} ≈ 10 nM). The C95A mutant receptor produced IPs following PAF stimulation, although its response curve was shifted to the right by 2 orders of magnitude, and did not reach a maximal response with the agonist concentrations used. The C95S mutant receptor displayed a subtle response only at 10^{-6} M PAF. As expected from the ligand binding results, the other mutants did not respond to PAF stimulation.

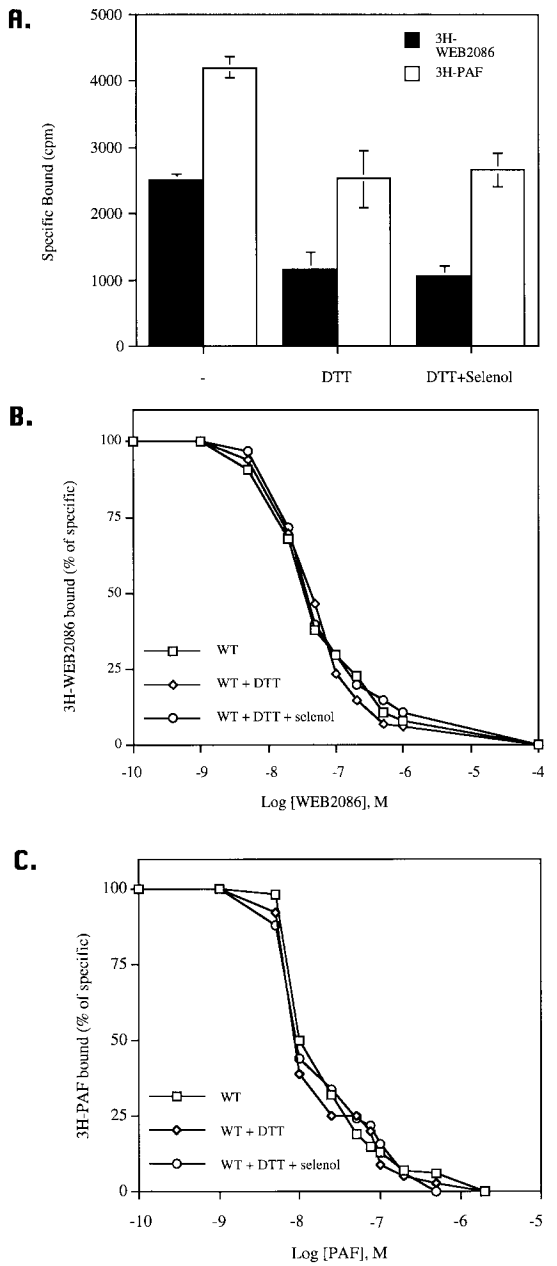

Fig. 2. Effects of DTT ± selenol. A: [^{3}H]WEB2086 and [^{3}H]PAF specific binding on CHO cells expressing the WT receptors. B: Competition binding isotherms of [^{3}H]WEB2086 by WEB2086. C: Competition binding isotherms of [^{3}H]PAF by PAF. The results are the means ± S.E.M. of three independent experiments, each done in triplicate.

3.3. Determination of mutant PAF receptor cell surface expression

The cmyc-tagged mutant and WT receptors were then analyzed for cell surface expression by flow cytometry. Fig. 4 illustrates that cells expressing the WT receptors showed 25 ± 5% of specific fluorescence associated with the presence of PAF receptors at the cell surface, whereas cells expressing mutant receptors C95A and C95S showed 4% and 6%, over basal fluorescence, respectively. Cys^{90} and Cys^{173} mutant receptors were apparently not expressed on the cell surface as they were not detected by flow cytometry (data not shown).

The possibility that the mutant receptors were produced but not expressed on the cell surface was examined using permeabilized cells and fluorescence microscopy. Fig. 5 shows that the WT PAF receptor is mostly found on the cell surface whereas the mutant receptors are all expressed and found in large quantity intracellularly. An isotype control antibody (WT-c) did not show any specific staining of wild type receptor expressing cells. Similar results with the control antibody were obtained with all the transfectants (results not illustrated).

Taken altogether, our results show that the Cys^{90}, Cys^{95} and Cys^{173} residues have profound effects on the structure-function relationship of the PAF receptor. The fluorescence and binding studies suggest that these amino acids might be necessary for proper protein folding of the receptor molecule. We have demonstrated that normal cell surface expression and ligand binding require these three residues. Whether bind-

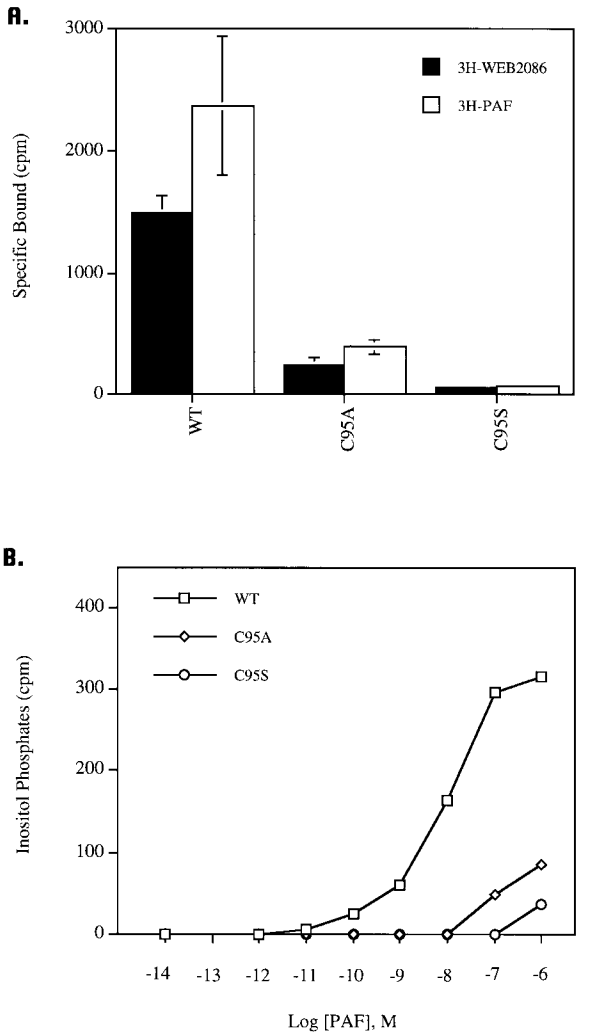

Fig. 3. Specific binding and IP production by the Cys^{95} mutants. A: [^{3}H]WEB2086 and [^{3}H]PAF specific binding on COS-7 cells expressing the WT, C95A and C95S mutant receptors. Reactions were carried out as described in Section 2. B: IP accumulation in response to graded concentrations of PAF. Total IPs were measured in COS-7 cells transfected with vector alone (control), the WT or the mutant receptors following a 30-s stimulation with the indicated PAF concentrations. The results are the means of three independent experiments, each done in duplicate.

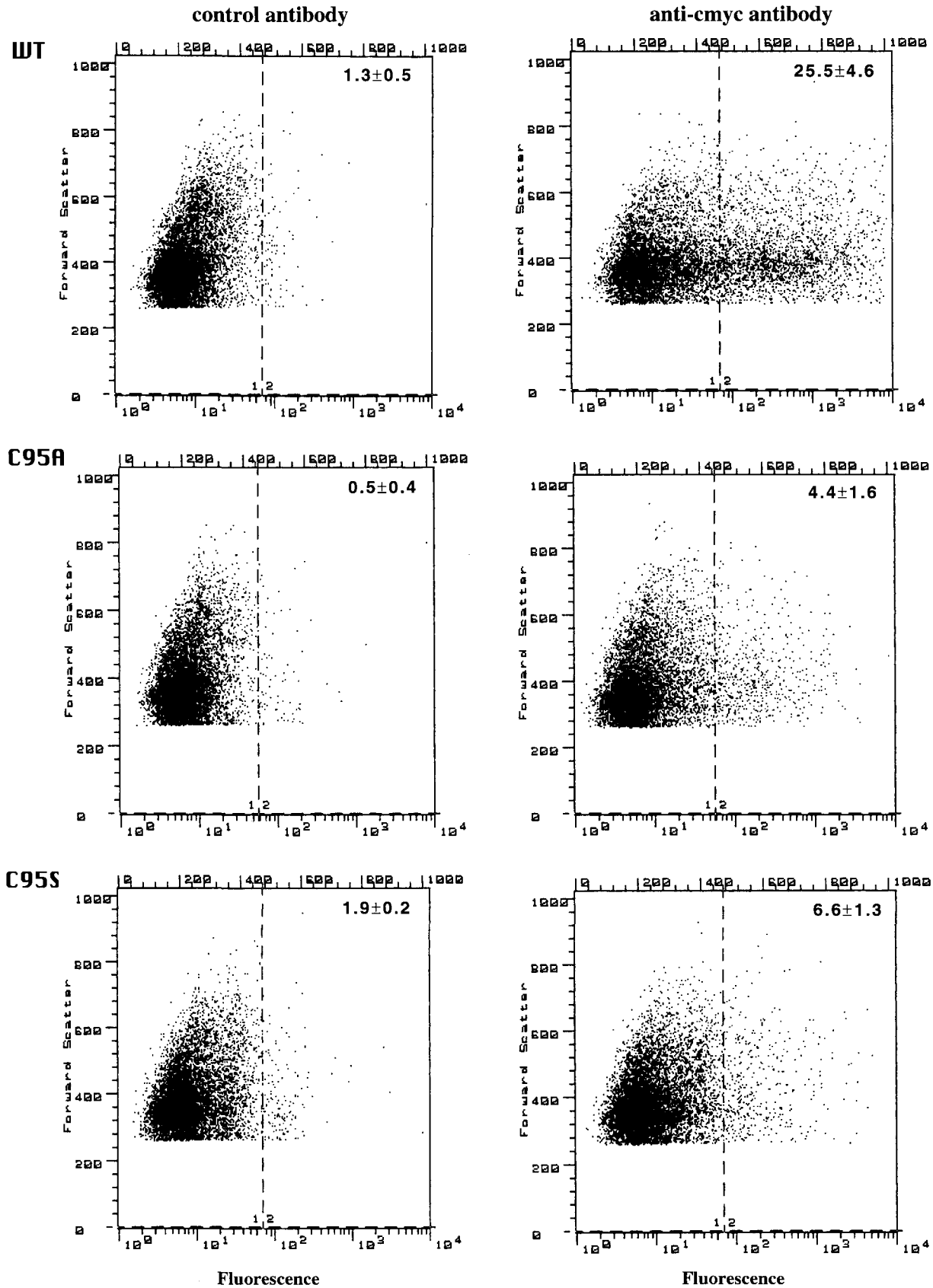

Fig. 4. Cytofluometry analysis of cmyc-tagged WT and Cys[95] mutant receptors transiently transfected in CHO cells. Transiently transfected CHO cells expressing the cmyc-tagged WT and mutant receptors were stained with a control antibody (anti-HA) or specific anti-cmyc antibody followed by a fluorescein-conjugated secondary antibody. The cells were then analyzed on a flow cytometer (FACScan). The dot plots illustrate a representative experiment of cells transfected with the WT receptor, C95A or C95S. The numbers are means ± S.E.M. of the specific % of fluorescence obtained from six independent experiments.

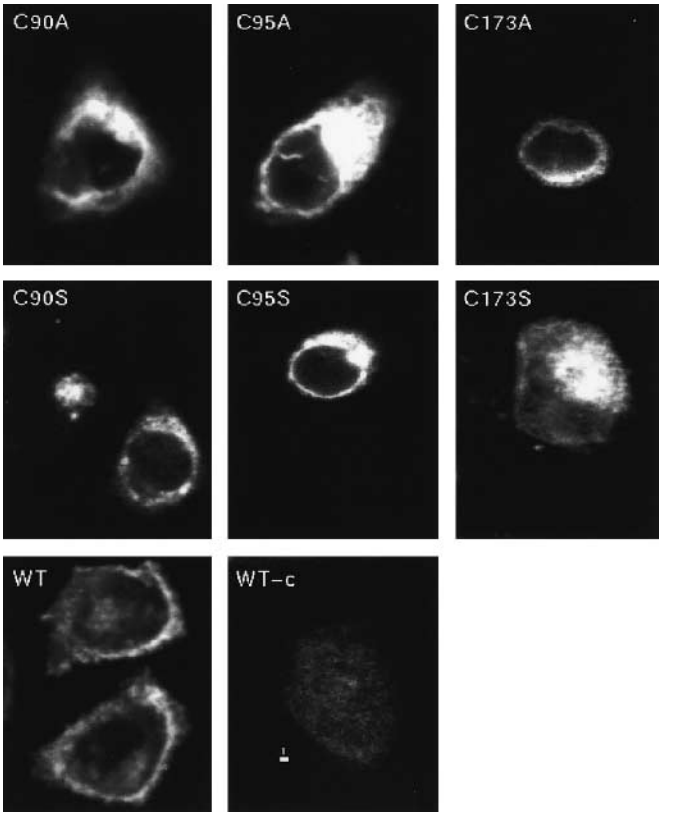

Fig. 5. Fluorescent microscopy analysis of the WT and cysteine mutant receptors. Expression of WT and mutant PAF receptors was examined in transiently transfected CHO cells. The photographs show a representative cell stained with anti-cmyc antibody and a fluorescein conjugated secondary antibody. WT-c shows a cell transfected with the WT receptor but stained with anti-HA as primary antibody, white bar represents 1 μm.

ing experiments were performed on crude membrane preparations or whole cells did not affect the results.

When either Cys^{90} or Cys^{173} were mutated to Ser or Ala, the resulting receptors were defective in ligand binding and were not detected on the cell surface. The C95A and C95S mutants displayed marginal ligand binding capability and some cell surface expression. Thus, it seems that the Cys^{90} and Cys^{173} would be involved in forming a disulfide bond together, since mutants from either site displayed the same phenotype. Cysteines at these respective positions are widely conserved throughout the superfamily of GPCRs and are felt to constitute a structurally necessary disulfide bond between extracellular loops one and two [18]. It also appears that elimination of this disulfide bond, by removing either of the two involved cysteines, destroys WEB2086 and PAF binding. These results would be in agreement with a recent molecular model of the PAF receptor [8]. Similar results were obtained on rhodopsin [19] with equivalent Cys (110 and 187) substitution to Ser. Mutant rhodopsins did not bind 11-*cis*-retinal, and were processed and inserted into membranes at relatively low levels. It was concluded that in absence of this disulfide bond, rhodopsin does not undergo proper protein folding. Mutation of the analogous cysteines at positions 98 and 178 of the M_1 muscarinic receptor [20] and of cysteines 105 and 183 of the thromboxane A_2 receptor [18], like the PAF receptor, resulted in a receptor with no binding or signaling capability. It is possible that the disulfide linkage may form during the early stages of receptor synthesis and be necessary for the normal folding and insertion into the membrane of these cell surface proteins. It has been shown that when participating cysteine residues are changed by mutagenesis, maturation and transport of the proteins from the ER are drastically reduced due to misfolding and aggregation [21]. This is supported by our data showing that the mutant receptors are produced abundantly but remain in the intracellular compartment.

From the binding and IP production data, the C95S and the C95A mutant receptors seem to have a lower affinity for PAF and/or a lower coupling efficiency to signal transduction molecules than the WT receptor, in addition to the very low level of cell-surface expression. Due to the impossibility to obtain binding isotherms for these mutants, it is not possible to determine if one or both mechanisms are specifically involved. The C95S and the C95A mutants also expressed different phenotypes, with the C95S receptor showing less specific ligand binding and a very subtle IP response to PAF stimulation in spite of an equivalent cell surface expression. Similarly, it was observed that substitution of Cys residues to Ser in rhodopsin resulted in more marked deleterious effects than substitution with Ala [22]. Our results suggest that Cys^{95} may be involved in formation of a disulfide bond with another Cys residue of the PAF receptor important to the structure-function relationship of the receptor, although less critical than the one possibly formed between Cys^{90} and Cys^{173}. Alternatively, mutation of Cys^{95} in itself may result in the phenotypes observed, suggesting a structural/functional role for a free sulfhydryl group of Cys^{95} in the PAF receptor. In fact,

data from alkylation studies have shown that free sulfhydryl groups were necessary for proper binding of PAF to its receptor [23].

In summary, the results of the present study demonstrate the involvement of disulfide linkages in the normal PAF receptor conformation. Moreover, we have shown that Cys^{90} and Cys^{173} appear to form a disulfide bond critical to the structure and function of the PAF receptor. Cys^{95} is also determinant in the native PAF receptor conformation, possibly by forming a disulfide bond with an amino acid of the receptor other than Cys^{173}, or by the requirement of its free sulfhydryl group. In the absence of these cysteines, the receptor protein is produced but not exported efficiently to the cell-surface membrane.

Acknowledgements: The authors would like to thank D. Gingras and S. Turcotte for excellent technical assistance. This work was supported by the Medical Research Council of Canada.

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