rin immunoprecipitation by SDS–polyacrylamide gel electrophoresis and autoradiography. The precipitation of clathrin by X19 IgG, from either A431 or CHO cells, was virtually indistinguishable (Fig. 2). Thus, X19 IgG appears to bind to human and Chinese hamster clathrin with similar affinity. In addition, A431 and CHO cells contain roughly equivalent amounts of clathrin.

To demonstrate unequivocally that X19 IgG could inhibit clathrin function in semi-intact CHO cells, we adapted the endocytosis assay (*8*) described above for use with CHO cells expressing human transferrin receptors (*10*). For this purpose, it was essential to grow the CHO cells on polylysine- or collagen-coated dishes to obtain semi-intact cells that could internalize ^{125}I-labeled transferrin in an ATP-dependent manner (*10*). X19 IgG inhibited the ATP-dependent endocytosis of transferrin by semi-intact CHO cells by as much as 65% when cells were scraped from polylysine-coated dishes (Table 2). Somewhat less inhibition (36%) was observed if cells were scraped from collagen-coated dishes; this is likely to reflect a difference in antibody accessibility to the cytoplasmic components in each type of broken cell. In summary, these data show that X19 IgG inhibits receptor-mediated endocytosis in broken CHO cells to a similar extent as that observed in broken human A431 cells (Table 1) or intact monkey kidney cells (*7*).

Clathrin-coated vesicles mediate fluid phase and adsorptive endocytosis. Clathrin is also thought to function at the TGN, in the diversion of lysosomal enzymes and secretory storage granule content away from constitutively secreted and membrane proteins bound for the cell surface (*11*). We have used antibodies that disrupt clathrin assembly, in conjunction with two different in vitro assays, to show that clathrin-coated vesicles appear not to carry man6P receptors from prelysosomes back to the TGN. This represents the first direct indication that in a transport cycle in which clathrin mediates the forward reaction (TGN export), another type of transport vesicle may be utilized for the reverse transport step (TGN import). In addition, since the transport of proteins from late endosomes to the TGN is a selective process (*2*), these data rule out a model in which the protein, clathrin, coats all transport vesicles involved in selective intracellular transport steps (*11*).

Our findings spotlight an interesting problem, namely, how clathrin-coated vesicle components recognize and capture man6P receptors at the TGN and not in prelysosomes. It has been proposed (*12*) that Golgi-specific "adaptin" proteins (*13*) sequester man6P receptors into clathrin-coated pits at the TGN. If this model is correct, our results imply that the adaptins (or adaptin-like proteins) must somehow be released from man6P receptors, either concurrent with the uncoating of these transport vesicles or after delivery of man6P receptors to their target organelle. In addition to proteins that accomplish cargo recognition, transport vesicles must also possess proteins that specify their organelle targets. Identification of the proteins that recognize man6P receptors in prelysosomes and direct them to the TGN represents an important challenge for the future.

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Molecular Localization of the Transforming and Secretory Properties of PDGF A and PDGF B

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Human platelet-derived growth factor (PDGF) is a connective tissue cell mitogen comprised of two related chains encoded by distinct genes. The B chain is the homolog of the v-*sis* oncogene product. Properties that distinguish these ligands include greater transforming potency of the B chain and more efficient secretion of the A chain. By a strategy involving the generation of PDGF A and B chimeras, these properties were mapped to distinct domains of the respective molecules. Increased transforming efficiency segregated with the ability to activate both α and β PDGF receptors. These findings genetically map PDGF B residues 105 to 144 as responsible for conformational alterations critical to β PDGF receptor interaction and provide a mechanistic basis for the greater transforming potency of the PDGF B chain.

HUMAN PLATELET-DERIVED GROWTH factor (PDGF) is a major mitogen for cells of connective tissue origin that is involved in development and wound healing (*1*). Abnormal expression of this growth factor has also been implicated in a variety of pathologic states including cancer (*2*). PDGF is a disulfide-linked dimer consisting of two related polypeptide chains, designated A and B, that are products of different genes. The gene encoding the human PDGF B chain is the normal counterpart of the v-*sis* oncogene (*3*). PDGF A and B chains are approximately 40% related (*4*) and contain eight conserved cysteine residues (*5, 6*). PDGF A and B chains can form homodimers as well as the AB heterodimer,

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and there is evidence for the natural occurrence of all three isoforms (*7*).

Although homodimers of either PDGF A or B are mitogenic as well as chemotactic for cells possessing the appropriate PDGF receptor (*8*), major differences in their biologic properties have been observed. The PDGF B chain gene exhibits 10- to 100-fold greater transforming efficiency in the NIH 3T3 transfection assay (*9*). Moreover, its product remains tightly cell associated (*10*), whereas the PDGF A chain is efficiently secreted (*9*). In addition, the two molecules differentially bind and activate the products of two distinct genes, encoding, respectively, the α and β PDGF receptor (*11, 12*). Whereas PDGF B interacts with either receptor, PDGF A binds and triggers only the α PDGF receptor (*8, 13*). In the present study, we constructed chimeras of PDGF A and B chains in an effort to map domains of each that potentially influence their normal functions and role in pathologic processes.

Chimeric constructs were developed with the use of preexisting or engineered (*14*) common restriction endonuclease sites within the PDGF A or PDGF B coding sequences. Each chimera was designated on the basis of the codon at which the recombination was performed. Four of the chimeric constructs, $A^{97}B^{99}$, $B^{98}A^{98}$, $A^{177}B^{179}$, and $B^{178}A^{178}$, were designed to maintain the functional integrity of the PDGF B minimal transforming domain (*15*) or the analogous region of PDGF A (Fig. 1). An additional six constructs further dissected the minimal transforming domain. The $A^{143}B^{145}$ and $B^{144}A^{144}$ chimeras divided the transforming region roughly in half, whereas $A^{104}B^{106}$ and $B^{105}A^{105}$ chimeras as well as $A^{153}B^{155}$ and $B^{154}A^{154}$ chimeras further subdivided the minimal transforming domain (Fig. 1).

All wild-type parental and recombinant PDGF constructs were transferred into a vector containing the metallothionein promoter (MMTneo) and analyzed for transforming activity by transfection of NIH 3T3 cells. Since the MMTneo vector also contained a dominant selectable neomycin marker gene, it was possible to score neomycin-resistant colony formation for each plasmid as well. Thus, we were able to precisely compare the specific transforming efficiencies of each construct.

The PDGF B expression vector had approximately 25-fold higher transforming efficiency than that of PDGF A (Fig. 1), as previously reported (*9*). The $A^{97}B^{99}$ and $B^{178}A^{178}$ chimeric constructs, which both contained the minimal transforming domain of the PDGF B gene product, had high transforming efficiency, similar to that of the wild-type PDGF B construct. In contrast, $B^{98}A^{98}$ and $A^{177}B^{179}$ chimeric constructs, which possessed the analogous domain of PDGF A, had low specific transforming efficiency, equivalent to that of PDGF A (Fig. 1). Chimeras $A^{104}B^{106}$, $B^{144}A^{144}$, and $B^{154}A^{154}$ also possessed high specific transforming efficiency, whereas the reciprocal chimeras, $B^{105}A^{105}$, $A^{143}B^{145}$, and $A^{153}B^{155}$, respectively, were only weakly transforming. Verification of our chimeric constructs was accomplished by subjecting each transfectant to immunoprecipitation analysis with antisera specific to PDGF A or PDGF B NH_2- or COOH-termini. In each case, the transfectants containing each chimera showed the expected PDGF A or PDGF B antigenic determinants and predicted intermediate sizes relative to PDGF A or PDGF B. All of these findings suggested that amino acid residues 105 to 144 of PDGF B were responsible for its more potent transforming activity (Fig. 1).

To test this hypothesis directly, we substituted only the minimal regions mapped above as being responsible for differences in transforming activities of the native PDGF A and B molecules. The $AB^{105-144}$ chimera possessed high specific transforming activity, indistinguishable from that of PDGF B (Fig. 1). Conversely, substitution of PDGF A codons 104 to 144 for those of PDGF B reduced transforming activity of the resulting chimera to that of the PDGF A molecule. Thus, the domain encompassed by PDGF B amino acid residues 105 to 144 was responsible for its more potent transforming properties.

Previous studies of the compartmentalization of PDGF A and PDGF B in transformed NIH 3T3 fibroblasts have shown that PDGF B remains tightly membrane associated, whereas PDGF A is efficiently secreted into culture fluids (*9*). No obvious structural motif such as hydrophobic stretches that might cause retention of PDGF B has been observed. Thus, we sought to identify the domains responsible for differences in secretion of the two molecules and whether such a domain could account for their different transforming potencies. After metabolic labeling of cultures for 4 hours, conditioned medium as well as a crude cell membrane fraction of each transfectant were subjected to immunoprecipitation analysis with a panel of PDGF antibodies. Crude membrane preparations of each transfectant showed roughly comparable levels of PDGF immunoreactive protein. However, only those chimeras that con-

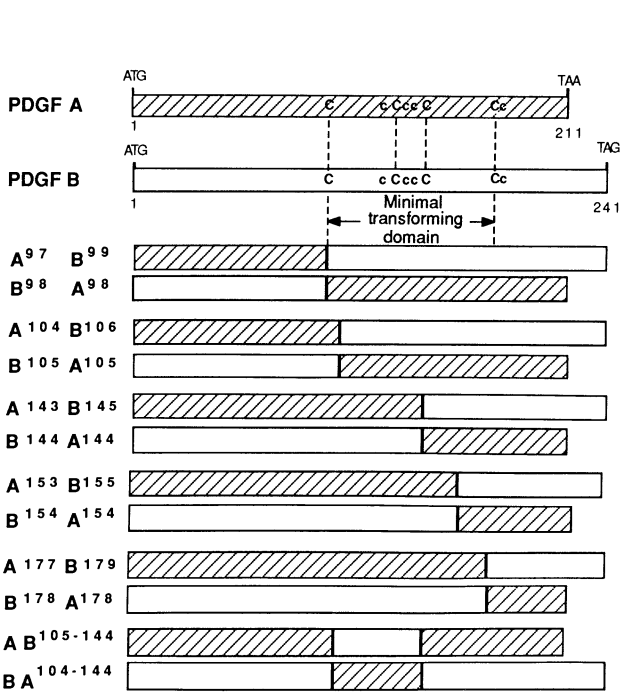

Fig. 1. Transforming activity of PDGF chimeric constructs. Chimeric PDGF molecules were constructed by recombination of PDGF A (hatched box) and B (open box) genes at a common preexistent or engineered (*15*) restriction endonuclease sites. The structure of each recombinant was verified by a combination of restriction endonuclease mapping and nucleotide sequence determination. NIH 3T3 cells were transfected with each recombinant DNA molecule by the calcium phosphate precipitation technique (*22*). Transfected cultures were either scored for colony formation in the presence of G418 (*23*) or for focus-forming activity as described (*9*). Data shown represent the mean values of three experiments. Relative transforming efficiency was calculated by dividing the number of foci (FFU) by the number of colonies (CFU) per nanogram of DNA for each construct relative to that obtained with PDGF A. Minimal transforming domain (*15*) and cysteine residues essential (C) or nonessential (c) for PDGF B transformation (*5*) are shown.

tained PDGF A COOH-terminal amino acid residues 178 to 211, namely $B^{98}A^{98}$, $B^{105}A^{105}$, $B^{144}A^{144}$, $B^{154}A^{154}$, and $B^{178}A^{178}$, were found to be efficiently secreted. The $B^{178}A^{178}$ chimera, which contained only PDGF A amino acid residues 178 to 211, was efficiently released, whereas the reciprocal chimera, $A^{177}B^{179}$, remained more than 90% membrane associated (Fig. 2).

To confirm our immunologic findings, we analyzed mitogenic activities associated with culture fluids and crude membrane preparations of transfectants containing parental or chimeric PDGF constructs. Comparable mitogenic activity was detected in each crude membrane fraction. However, only in the case of PDGF A and those chimeras containing at least the COOH-terminal 34 amino acid residues of PDGF A was mitogenic activity detectable in culture fluids. In each case, the mitogenic activity was specifically inhibited by neutralizing PDGF antibody, establishing the PDGF-related nature of the secreted mitogen. Again, potent transforming activity mapped to PDGF B amino acid residues 105 to 144 (Fig. 1). Thus, localization of the domain responsible for differences in PDGF A and B secretory properties to their COOH-terminal regions excluded this property as being responsible for their different transforming activities.

We next investigated whether PDGF receptor binding or activation, or both, might be responsible for the differences in oncogenic potency of PDGF A and B. We examined the steady-state level of tyrosine phosphorylation of α and β receptors expressed in NIH 3T3 transfectants containing either wild-type PDGF A or B constructs as well as each of the chimeras. To do so, cell lysates were enriched for each receptor by immunoprecipitation with α or β PDGF receptor–specific peptide antisera followed by immunoblotting with antibody to phosphotyrosine. As shown in Fig. 3, NIH 3T3 cells showed no detectable α or β PDGF receptor tyrosine phosphorylation. As expected from known receptor binding properties of each ligand (*8, 13*), NIH 3T3 cells that expressed PDGF A were phosphorylated at tyrosine in the 180-kD α but not β PDGF receptor species. In contrast, both 180 kD α and β PDGF receptor species were tyrosine phosphorylated in PDGF B–producing cells (Fig. 3). The specificity of the antibody was demonstrated by the ability of phosphotyrosine but not phosphoserine or phosphothreonine to compete for immunodetection of these proteins.

When the steady-state level of PDGF receptor tyrosine phosphorylation was examined in transfectants containing the PDGF chimeras, readily detectable levels of the activated 180-kD α PDGF receptor were observed in each case (Fig. 3). However, there was chronic activation of the 180-kD β PDGF receptor species of cells expressing $A^{97}B^{99}$, $A^{104}B^{106}$, $B^{144}A^{144}$, $B^{154}A^{154}$, as well as $B^{178}A^{178}$ chimeras, all of which contained at least PDGF B amino acid residues 105 through 144. The $AB^{105-144}$ chimera, which substituted only PDGF B amino acid residues 105 through 144 into the analogous region of PDGF A, had the same pattern of receptor tyrosine phosphorylation as observed with PDGF B (Fig. 3). Conversely, the switch of analogous PDGF A amino acid residues into PDGF B led to a pattern of receptor tyrosine phosphorylation indistinguishable from that of PDGF A. These results show that amino acid residues 105 through 144 are responsible for the ability of PDGF B to preferentially bind and activate the β PDGF receptor and genetically map the increased transforming activity of PDGF B molecule to these same residues.

Whereas all chimeras activated the α PDGF receptor, there was a complete correlation between increased transforming efficiency and the ability to activate β PDGF receptors as well. The domain of PDGF B that was responsible for these findings was genetically mapped to encompass codons 105 to 144. Immunochemical studies have shown that this domain contains a surface epitope important for PDGF receptor binding and activation (*16*). Our present studies establish that this domain is not only critical to PDGF receptor triggering, but is the major determinant of subtle conformational differences that specify interaction of PDGF B with the β PDGF receptor.

We also mapped an independent domain responsible for the differences in PDGF A and B secretory properties to the COOH-terminal amino acid residues of PDGF A or PDGF B. These findings indicate that either the PDGF B COOH-terminus causes its preferential cell association or the analogous domain of PDGF A promotes its secretion. The former seems less likely since normal processing of PDGF B leads to removal of its COOH-terminus at the cell surface, without significant release into culture fluids (*10, 17*).

The quantitative differences in transforming activities of PDGF A and B chain genes for NIH 3T3 cells in vitro correlate with in vivo findings that a retrovirus encoding PDGF B induces fibrosarcomas in nude mice (*18*), whereas an analogous retrovirus encoding PDGF A has as yet not produced detectable tumors (*19*). Differences have been observed in the relative abilities of

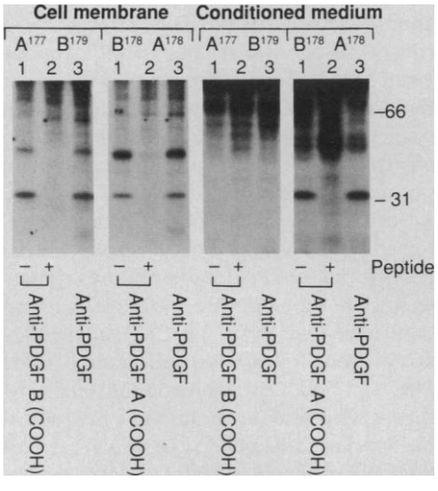

Fig. 2. Compartmentalization of PDGF chimeric constructs. NIH 3T3 cells transfected with $A^{177}B^{179}$ or $B^{178}A^{178}$ were metabolically labeled as described (*10*). Crude cellular membranes or conditioned media were examined by immunoprecipitation with the antibodies indicated. In some cases, antibodies were incubated with excess homologous peptide (lanes 2). Immune complexes were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions, and results were visualized by fluorography for 12 days (cell membrane) or 36 hours (conditioned medium).

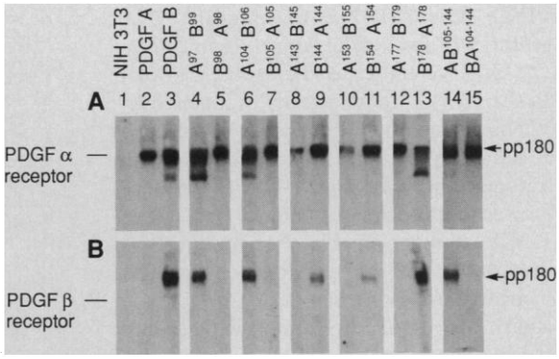

Fig. 3. Tyrosine phosphorylation of α and β PDGF receptors in NIH 3T3 cells expressing PDGF chimeras. NIH 3T3 cells (lane 1) or transfectants expressing PDGF A (lane 2), PDGF B (lane 3), or the chimeric PDGF constructs (lanes 4 through 15) were incubated overnight in Dulbecco's modified Eagle's medium containing 25 μM $ZnCl_2$. After 16 hours, cells were washed with phosphate-buffered saline and 1.0 mM sodium orthovanadate, and lysed as described (*8*). Protein extracts were immunoprecipitated with antibodies specific for α (**A**) or β (**B**) PDGF receptor peptides (*8, 11*). Immunoprecipitated proteins were blotted to Immobilon-P and probed with an anti-phosphotyrosine specific antibody (*8, 11*). Filters were treated with ^{125}I-labeled protein A and subjected to autoradiography. The electrophoretic mobility of pp180 α and β PDGF receptors are shown. In some cases immature forms of PDGF receptors were recognized as well.

PDGF A or PDGF B to stimulate DNA synthesis in a variety of fibroblasts in culture (*20, 21*). We and others have observed that PDGF B is a more potent mitogen than PDGF A for NIH 3T3 cells (*21*). Yet, both PDGF receptors can effectively couple with mitogenic signaling pathways (*8*). Thus, the greater transforming potency of those PDGF chimeras capable of triggering β as well as α PDGF receptors likely reflects increased levels of receptor activation. The ability to further localize amino acid residues specifically required for β PDGF receptor binding or activation, or both, should aid in efforts to develop antagonists of β PDGF receptor function.

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Design and Synthesis of a Peptide Having Chymotrypsin-Like Esterase Activity

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A peptide having enzyme-like catalytic activity has been designed and synthesized. Computer modeling was used to design a bundle of four short parallel amphipathic helical peptides bearing the serine protease catalytic site residues serine, histidine, and aspartic acid at the amino end of the bundle in the same spatial arrangement as in chymotrypsin (ChTr). The necessary "oxyanion hole" and substrate binding pocket for acetyltyrosine ethyl ester, a classical ChTr substrate, were included in the design. The four chains were linked covalently at their carboxyl ends. The peptide has affinity for ChTr ester substrates similar to that of ChTr and hydrolyzes them at rates ~0.01 that of ChTr; total turnovers >100 have been observed. The peptide is inhibited by ChTr specific inhibitors and is inactive toward benzoyl arginine ethyl ester, a trypsin substrate. The peptide is inactivated by heating above 60°C, but recovers full catalytic activity upon cooling and lyophilization from acetic acid.

THE DESIGN AND SYNTHESIS OF MOLecules having catalytic activity and substrate specificity resembling that of natural enzymes has long been a goal of chemists (*1*). Some nonprotein organic molecules such as cyclodextrins and paracyclophanes show some enzyme-like characteristics. Recently monoclonal antibodies that catalyze specific reactions have been evoked by immunization with molecules that resemble sterically the transition state intermediate of the substrate being transformed (*2*). Although progress has been made in design and synthesis of peptides that assume a desired conformation in solution (*3–5*), the step of adding an enzyme-like constellation of catalytic amino acid residues to these synthetic molecules has not been previously accomplished. We report the design and synthesis of a 73-residue peptide "chymohelizyme-1" (CHZ-1) that shows characteristics resembling those of ChTr.

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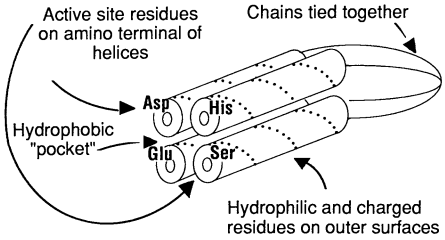

Fig. 1. Generic structure of a "chymohelizyme."

The mammalian serine proteases, of which ChTr is a much-studied example, possess a "catalytic triad" of amino acid residues so positioned as to confer hydrolase activity on the protein. In ChTr (which is synthesized as a single chain of 245 amino acid residues), these are His^{57}, Asp^{102}, and Ser^{195}. The His and Asp residues confer special reactivity on the hydroxyl group of Ser^{195}; the oxygen of this hydroxyl group attacks the electropositive carbonyl carbon of the substrate. A portion of the protein backbone chain (at Gly^{193} and Ser^{195}) is so positioned as to form strong hydrogen bonds to the tetrahedral oxyanion intermediate of the carbonyl group of the peptide or ester bond being broken; this binding site constitutes the so-called "oxyanion hole" of the enzyme (*6, 7*). The bacterial protease subtilisin possesses the same three catalytic amino acid residues positioned in space in a totally analogous way, but the 274-residue chain of this protein shows no homology to that of the mammalian serine proteases, suggesting that very different structures can effectively hold the active site residues in position for catalysis.

Substrate specificity of enzymes is determined by the arrangement of groups in the enzyme that bind structural features characteristic of the substrate molecule. Protein chains are hydrolyzed by ChTr at the carboxyl group of the aromatic amino acids Phe, Tyr, and Trp. Aromatic side chains of these amino acids are bound in a hydrophobic "pocket" adjacent to the catalytic triad; this pocket is composed of amino acid hydrophobic side chains and is approximately perpendicular to the plane of the catalytic triad residues. Esters and amides of aromatic amino acids having blocked amino groups are hydrolyzed by ChTr. Acetyltyrosine ethyl ester (ATEE), benzyloxycarbonyltyrosine *p*-nitrophenyl ester (ZTONP), and benzoyltyrosine ethyl ester (BTEE) are convenient ChTr substrates.

Given the recent great increase in understanding of the features in peptides that