activity which would make it a possible target for therapeutic interventions[28]. □

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Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160

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THE envelope glycoprotein of human immunodeficiency virus (HIV) initiates infection[1] by mediating fusion of the viral envelope with the cell membrane. Fusion activity requires proteolytic cleavage of the gp160 protein into gp120 and gp41 at a site containing several arginine and lysine residues[2]. Activation at basic cleavage sites is observed with many membrane proteins of cellular and viral origin. We have recently found that the enzyme activating the haemagglutinin of fowl plague virus (FPV), an avian influenza virus, is furin[3]. Furin, a subtilisin-like eukaryotic endoprotease[4–6], has a substrate specificity for the consensus amino-acid sequence Arg-X-Lys/Arg-Arg at the cleavage site[7]. We show here that the glycoprotein of HIV-1, which has the same protease recognition motif as the FPV haemagglutinin, is also activated by furin.

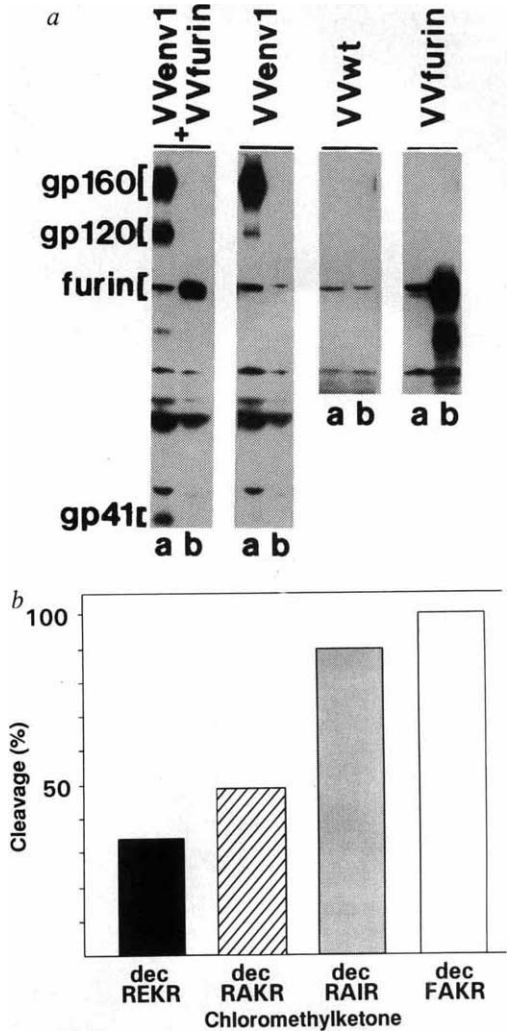


FIG. 1 Cleavage of the HIV-1 glycoprotein by human furin expressed from a vaccinia virus vector and its inhibition by sequence-specific peptidyl-chloromethylketones. *a*, Coexpression of furin and gp160 by recombinant vaccinia viruses. Proteins immunoprecipitated with anti-HIV serum (a) or anti-furin serum (b) from lysates of CV-1 cells are shown. Cells were doubly infected with recombinant vaccinia viruses VVenvl[8] and VVfurin[5] or singly infected with each recombinant and wild-type vaccinia virus (VVwt). *b*, Cleavage inhibition by peptidyl chloromethylketones (cmk). The effect of the inhibitors decanoyl-arginyl-glutamyl-lysyl-arginyl-choromethylketone (dec-REKR), decanoyl-arginyl-alanyl-lysyl-arginyl-chloromethylketone (decRAKR), decanoyl-arginyl-alanyl-isoleucyl-arginyl-chloromethylketone (decRAIR), and decanoyl-phenylalanyl-alanyl-lysyl-arginyl-chloromethylketone (decFAKR; H.A., R. Wilkstrom, E.S., W. Bennan and R. S. Fuller, manuscript submitted) were analysed at a concentration of 50 μM in cells coinfected with VVenv1 and VVfurin.
METHODS. Multiplicities of infection (m.o.i.) were 5 PFU per cell. At 19 h post infection (p.i.), cells were starved of methionine and cysteine for 1 h and then labelled with [^{35}S]methionine and [^{35}S]cysteine for 2h. After a 3-h chase period, cells were lysed in 1 ml lysis buffer[3]. Equal volumes of the cellular lysates were exposed for 1 h at 4° to either pooled sera of healthy HIV-1 seropositive donors (anti-HIV antiserum[15]; NIH AIDS Research and Reference Reagent Program, Rockville, MD) or a rabbit antiserum against furin. Antisera were used undiluted in all experiments to accomplish quantitative immunoprecipitation. Protein A–Sepharose beads (Sigma) were then added for 2 h at 4 °C. Precipitates were analysed by electrophoresis on 8% polyacrylamide gels in the presence of SDS followed by fluorography. Cleavage inhibitors were added 1 h and replenished at 10 h p.i.[16]. Control samples were incubated without inhibitors. After SDS–PAGE and fluorography, the extent of cleavage was determined as follows: radioactive bands were cut out of the gel, rehydrated in water and then solubilized in Soluene-350 (Packard) for 1 h at 60 °C and counted in liquid scintillator (Zinsser Analytic). The extent of cleavage was determined as the amount of gp120 observed in the presence of inhibitors relative to the amount of gp120 observed in the absence of these inhibitors.

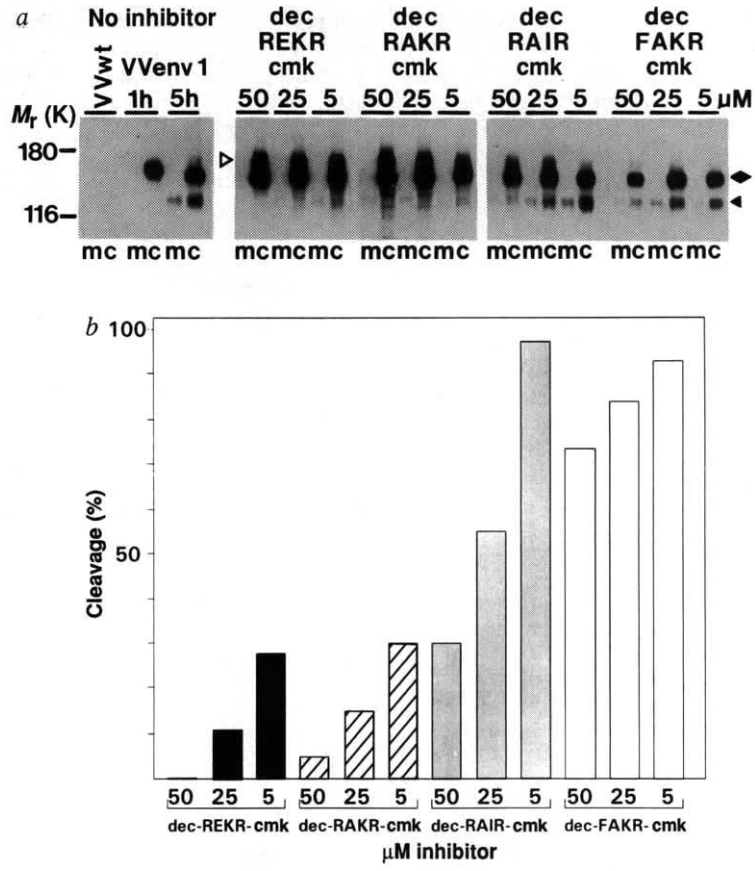


FIG. 2 Effects of peptidyl-chloromethylketones on gp160 cleavage mediated by the endogenous protease of HeLa cells. *a*, Inhibition of cleavage. HIV glycoprotein immunoprecipitated from medium (m) and cell lysates (c) was analysed. Cells incubated without inhibitors were either lysed immediately after the pulse (1 h) or after a 5-h chase period. Cells incubated with inhibitors were analysed only after pulse-chase labelling. Open arrowhead indicates gp160 with mature carbohydrate side chain; ◆, gp160 with immature carbohydrate side chains; filled arrowheads, gp120. HeLa T4 cells were infected with either VVwt or VVenv1 at m.o.i. of 10. *b*, Quantification of cleavage inhibition. The extent of cleavage in the samples shown in *a* is measured as the amount of gp120 observed in the presence of inhibitors relative to the amount of gp120 observed in the absence of inhibitors.
METHODS. After a 1-h adsorption period, DMEM containing various concentrations of the different inhibitors was added. At 10 h p.i., cells were starved of methionine and cysteine for 1 h and subsequently exposed to pulse-chase labelling with [^{35}S]methionine and [^{35}S]cysteine. HIV glycoprotein was immunoprecipitated with human anti-HIV antiserum from media and cell lysates and analysed by SDS–PAGE.

Furthermore, we present evidence that peptidyl-chloromethyl ketones that have the Arg-X-Lys/Arg-Arg motif and which are specific inhibitors of furin[3] interfere with cleavage of the HIV glycoprotein and hence its activation and the formation of infectious virus particles.

To determine whether cleavage of the HIV-1 precursor glycoprotein gp160 is mediated by furin, gp160 complementary DNA[8] and human furin cDNA isolated from a hepatoma (HepG2) cDNA library[5] were expressed in CV-1 cells alone or in combination using vaccinia virus recombinants as vectors (Fig. 1). Previous studies on the expression of nerve growth factor β-NGF[5] and on FPV haemagglutinin[3] had shown that late after infection with the vaccinia vectors, cleavage by the endogenous CV-1 protease ceased and the expression products accumulated in the uncleaved form. Similarly, only gp160 was observed when cells were labelled at 19h after infection with VVenv1 alone, but after coexpression with human furin, which is a protein of M_r92,000 (92K)[3], the cleavage products gp120 and gp41 were detected. Therefore furin is able to process the HIV-1 glycoprotein. The finding that only ~40% of gp160 is cleaved can be explained by the observation that a relatively high proportion of the glycoprotein is retained in the endoplasmic reticulum, presumably as a result of incorrect folding[9]. Thus, only a minority of gp160 molecules appear to be able to reach the Golgi complex of the *trans* Golgi compartment, where gp160 cleavage is supposed to occur[10–12] and where furin has been localized[5]. Figure 1*b* shows that chloroalkylketones with an appropriate peptide moiety[3] (decanoyl-REKR-cmk and decanoyl-RAKR-cmk; one-letter amino-acid code) interfere with the cleavage of gp160 by overexpressed furin, whereas compounds not having the correct consensus sequence (decanoyl-RAIR-cmk and decanoyl-FAKR-cmk) are inactive. Therefore specific inhibitors of gp160 cleavage are now available.

To test whether peptidyl-chloromethylketones are able to inhibit endogenous furin present in human cells, their effect on HIV-1 glycoprotein expressed in HeLa–CD4 cells by a vaccinia virus vector was analysed (Fig. 2*a*, *b*). When pulse-chase labelled at 10 h post-infection cleavage of gp160 and release of gp120 into the culture medium can be clearly detected. In the presence of 5 µM decanoyl-REKR-cmk and decanoyl-RAKR-cmk, cleavage is inhibited and the precursor that is not released into the medium has acquired a higher electrophoretic mobility. As the precursor is resistant to endoglucosaminidase H under these conditions, the higher molecular weight probably reflects

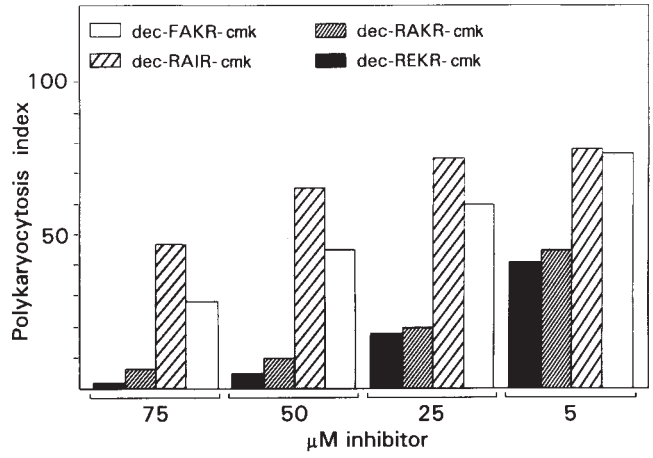
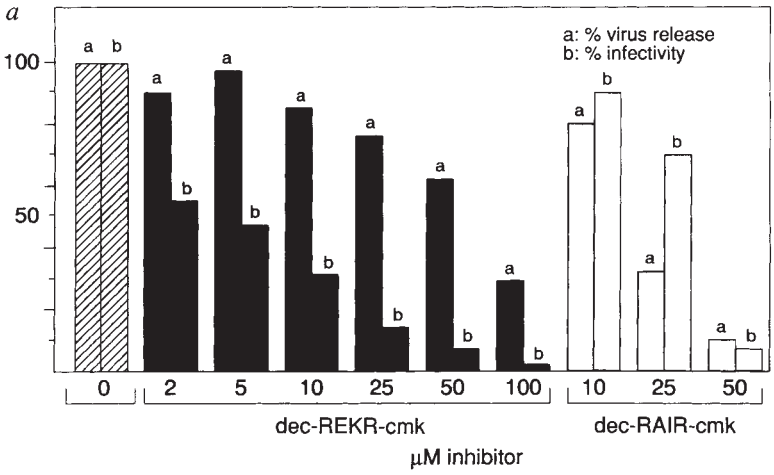


FIG. 3 Effect of peptidyl-chloromethylketones on HIV glycoprotein-induced syncytia formation in HeLa T4 cells. Confluent monolayers of HeLa T4 cells were infected with either VVwt or VVenv1 at m.o.i. of 2.5. After 60-min adsorption, viral inoculum was removed and medium containing inhibitors was added. At 8 h p.i. cell cultures were examined by phase-contrast microscopy (magnification, 250×). The polykaryocytosis indices (PI) (percentage of total nuclei of a monolayer present in polykaryons) are indicated by columns. PI of VVenv1 infected cells without inhibitor was ≥80; PI of VVwt infected cells was <5.

maturation of the oligosaccharides to more elongated forms. Decanoyl-RAIR-cmk and decanoyl-FAKR-cmk do not show these effects. These findings support the concept that cleavage of gp160 is specifically blocked by furin inhibitors, and they suggest that the inhibitors do not interfere with other processing events (see below).

To elucidate the effects of cleavage inhibition on biological functions of the envelope glycoprotein, we first analysed syncytia formation in HeLa-CD4 cells. Figure 3 shows the inhibitory potential of the compounds. Again there was a distinct effect with decanoyl-RAKR-cmk and decanoyl-REKR-cmk, which completely blocked cell fusion at $\geq 50\,\mu M$. The other compounds barely inhibited fusion. Decanoyl-FAKR-cmk, as compared to decanoyl-RAIR-cmk, had retained some inhibitory activity which, however, seems to reflect the relative toxicity of this compound (compare Fig. 2*a*) and is therefore probably not specific. To test whether the inhibitors also interfered with virus infectivity, we analysed the effects of decanoyl-REKR-cmk and decanoyl-RAIR-cmk on the replication of HIV-1 in human lymphocytes. With increasing doses of decanoyl-REKR-cmk, virion release decreases only slightly, whereas infectivity drops sharply. Thus, at 50 μM decanoyl-REKR-cmk, the infectivity of the released virus is about 10-fold lower than that of untreated control virus. In contrast, in the presence of decanoyl-RAIR-cmk, there is a drastic reduction in released particles, but their infectivity is not reduced. These observations again underline the potential of decanoyl-REKR-cmk as a specific inhibitor of proteolytic activation, whereas decanoyl-RAIR-cmk shows not only less specificity but also considerable toxicity. Viral glycoproteins present in MT-4 cells (Fig. 4*b*) and virus particles (Fig. 4*c*) were analysed after inhibitor treatment. In agreement with the data shown in Fig. 2*a*, there is an increase in the gp160:gp120 ratio in the cell lysates with increasing decanoyl-REKR-cmk concentrations. The increased molecular weight of gp160, which presumably reflects carbohydrate maturation (Fig. 2*a*), can also be seen. With the dibasic compound decanoyl-RAIR-cmk, the gp160:gp120 ratio remains virtually constant and the larger form of gp160 does not appear. This again



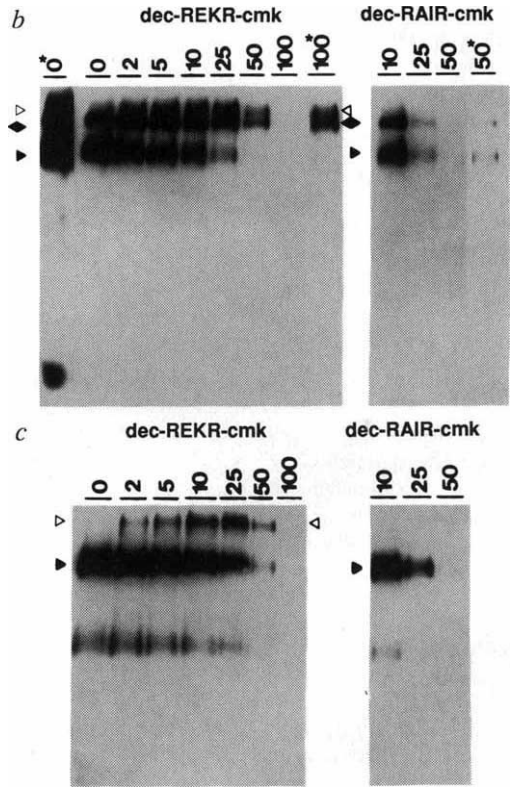


FIG. 4 Effects of cleavage inhibitors on formation of infectious virus. Formation of HIV-1 particles (strain HTLV-IIIB; ref. 17 was examined in the transformed T-lymphocyte cell line MT-4 (ref. 18) *a*, Infectivity of virus released from inhibitor-treated MT-4 cells. Using an ELISA test, it was established that in the inhibitor-treated cultures 50–80% of the total antigen in the medium was precipitable by polyethylene glycol and so are virus particles[19]. This indicates that there has been no significant release of soluble antigen from the cultures as a result of cell damage, so we interpreted the decrease in HIV-1 released into the medium at higher inhibitor concentrations as a measure of the general cytotoxic effect of such inhibitors on MT-4 cells. The infectivity of virus particles released from inhibitor-treated cultures was analysed by infecting fresh MT-4 cells with equal amounts of virus as determined by ELISA. *b*, Viral glycoproteins in inhibitor-treated MT-4 cells and *c*, in released virus particles. The positions of the viral glycoproteins gp160 (◆), gp120 (▲), gp41 (■), and gp160 with mature oligosaccharide side chains (△) are indicated. An immunoprecipitated component observed in the virus, migrating at about 75K, presumably represents a proteolytic cleavage product of gp120.0*, 100* (decREKR-cmk) and 50* (decRAIR-cmk) indicate longer exposures of lanes 0, 100 (decREKR-cmk) and 50 (decRAIR-cmk), respectively. The amount of labelled glycoprotein decreases with increasing inhibitor concentration. A part of this decrease can be accounted for by the decrease in the amount of released HIV-1 with increasing inhibitor concentration, see *a*. But at the highest concentrations of both inhibitors, the reduction in the amount of labelled glycoprotein is higher than the reduction of released virus. This may indicate a differentially greater effect on the viral glycoproteins.

METHODS. Rapid synchronous replication was achieved by adding 5% of a completely infected MT-4 culture (at 3 days p.i.) to 95% fresh subconfluent MT-4 cells, cultivated in RPMI 1640 medium. At 20 h p.i. the infected cells were plated in 1-ml aliquots and different concentrations of inhibitors added; 6 h later cells were collected by low-speed centrifugation, the medium containing virus that had been synthesized before inhibitor treatment was discarded, and the cells were resuspended in 1 ml medium containing [^{3}H]glucosamine (100 μCi ml^{-1}; Amersham–Buchler). Inhibitors were replenished 7 h later. After a further 8 h, cells and media were collected and prepared for further analysis. Immunofluorescence using human AIDS serum revealed that all cells were infected. Infectivity assay of released virus: virus particles from 800 μl medium were precipitated with polyethylene glycol (PEG) as described[20]. The amount of HIV antigen in the redissolved PEG pellets and in the original culture medium without PEG were determined using a standard p24-capture ELISA (Organon Teknika)[21]. Equal amounts of virus particles, as determined by ELISA of the PEG pellets from media of inhibitor-treated cultures, were used to infect fresh subconfluent MT-4 cells. At 5 h p.i., infected cultures were washed with medium to remove input virus and cultured further. At 31 h p.i., the amounts of newly synthesized virus released into the medium were determined again by ELISA. These values reflect the infectivity of the virus used for infection. The amount and infectivity of virus released from untreated cultures were each taken as 100%. Analysis of envelope glycoprotein: labelled cells and PEG-precipitated virus, both from 800 μl culture treated with inhibitors at micromolar concentrations, were dissolved in lysis buffer (1% Triton-X100, 0.5% desoxycholate, 0.1% SDS, 2mM EDTA in 10 mM sodium phosphate, pH 7.2) and immunoprecipitated with rabbit antiserum specific for gp120 and gp160, followed by polyacrylamide gel electrophoresis[22].

indicates that, unlike decanoyl-REKR-cmk, decanoyl-RAIR-cmk does not specifically affect proteolytic cleavage of gp160. Virus particles released from decanoyl-REKR-cmk-treated cells (Fig. 4*c*) contain increasing amounts of gp160 in its high-M_r form. Whether or not gp160 is incorporated into virions has been the subject of some controversy[13,14]. The data presented here demonstrate that cleavage is not a prerequisite for the transport of the glycoprotein to the cell surface and its incorporation into virions. On the other hand, the reduced infectivity of virus produced in the presence of decanoyl-REKR-cmk is clearly a consequence of the inability of gp160 to induce fusion. The virus produced under these conditions still contained some gp120, indicating that cleavage inhibition was not complete. As cleavage is a late step in processing, it is not surprising that the proportion of cleaved glycoprotein was higher in virions than in cells. The coexistence of gp120 and gp160 in virus particles raises the possibility that cleaved and hence potentially functional glycoprotein may be negatively influenced by an association with gp160 to hetero-oligomers.

As decanoyl-REKR-cmk and decanoyl-RAKR-cmk are specific inhibitors of furin[3], their interference with cleavage of gp160 supports the results obtained after expressing the enzyme from cDNA in identifying it as the activating protease. Our data show also that the inhibitors reduce the infectivity of the virus and may therefore have the potential to arrest the spread of infection in the organism. Furthermore, the inhibitors prevent shedding of gp120 from the surface of infected cells, which is also suspected to play a role in viral pathogenesis. Further investigation will establish whether peptidyl-chloromethylketones that mimic the cleavage site of gp160 can be effective as therapeutic agents against HIV infection. □

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Natural inhibitor of transforming growth factor-β protects against scarring in experimental kidney disease

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THE central pathological feature of human kidney disease that leads to kidney failure is the accumulation of extracellular matrix in glomeruli. Overexpression of transforming growth factor-β (TGF-β) underlies the accumulation of pathological matrix in experimental glomerulonephritis[1]. Administration of an antibody raised against TGF-β to glomerulonephritic rats suppresses glomerular matrix production and prevents matrix accumulation in the injured glomeruli[2]. One of the matrix components induced by TGF-β, the proteoglycan decorin, can bind TGF-β and neutralize its biological activity[3], so decorin may be a natural regulator of TGF-β (refs 3, 4). We tested whether decorin could antagonize the action of TGF-β *in vivo* using the experimental glomerulonephritis model[1]. We report here that administration of decorin inhibits the increased production of extracellular matrix and attenuates manifestations of disease, confirming our hypothesis. On the basis of our results, decorin may eventually prove to be clinically useful in diseases associated with overproduction of TGF-β.

Three isoforms of TGF-β are expressed in mammals, TGF-β1, 2 and 3 (ref. 5). As shown in Fig. 1, recombinant human decorin blocks the growth-inhibiting action of each TGF-β isoform in

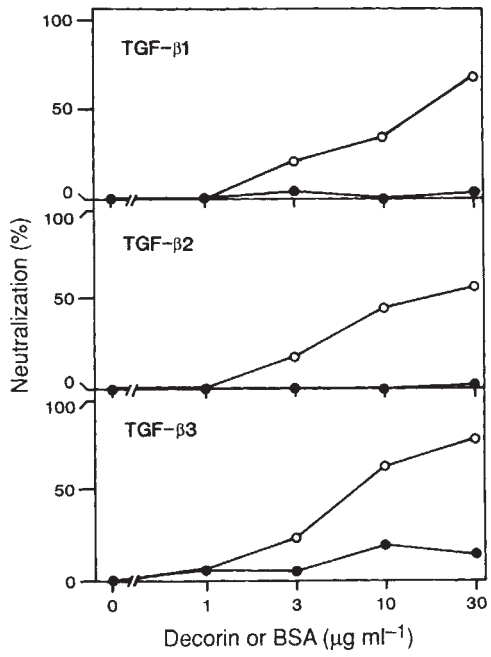

FIG. 1 Neutralization of the activity of TGF-β1, 2 and 3 by recombinant human decorin; TGF-β inhibits mink lung cell growth. [^{3}H]Thymidine incorporation was determined with TGF-β alone (not shown) and with increasing concentrations of decorin (circles) or bovine serum albumin (BSA) (filled circles) as described[3]. Incorporation without TGF-β or decorin is defined as 100% (125,000 c.p.m. for TGF-β1 and TGF-β3; 107,000 c.p.m. for TGF-β2); incorporation with TGF-β alone is defined as 0% (57,000 c.p.m. for TGF-β1; 78,000 c.p.m. for TGF-β2; 59,000 c.p.m. for TGF-β3). Points represent means of duplicate samples.
METHODS. Purified human TGF-β1 and 2 were from R & D Systems (Minneapolis, MN); TGF-β3 was a gift from A. Roberts and M. Sporn. TGF-β was added at concentrations that inhibited [^{3}H]thymidine incorporation by 50%; 0.2 ng ml^{-1}, 0.1 ng ml^{-1} and 0.05 mg ml^{-1} for TGF-β1, TGF-β2 and TGF-β3, respectively. Recombinant decorin and BSA were prepared as described for Fig. 2.