The EMBO Journal Vol.17 No.20 pp.5948–5963, 1998

Pathogenic poxviruses reveal viral strategies to exploit the ErbB signaling network

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Virulence of poxviruses, the causative agents of smallpox, depends on virus-encoded growth factors related to the mammalian epidermal growth factor (EGF). Here we report that the growth factors of Shope fibroma virus, Myxoma virus and vaccinia virus (SFGF, MGF and VGF) display unique patterns of specificity to ErbB receptor tyrosine kinases; whereas SFGF is a broad-specificity ligand, VGF binds primarily to ErbB-1 homodimers, and the exclusive receptor for MGF is a heterodimer comprised of ErbB-2 and ErbB-3. In spite of 10- to 1000-fold lower binding affinity to their respective receptors, the viral ligands are mitogenically equivalent or even more potent than their mammalian counterparts. This remarkable enhancement of cell growth is due to attenuation of receptor degradation and ubiquitination, which leads to sustained signal transduction. Our results imply that signal potentiation and precise targeting to specific receptor combinations contribute to cell transformation at sites of poxvirus infection, and they underscore the importance of the often ignored low-affinity ligand–receptor interactions.

Keywords: DNA virus/growth factor/oncogene/signal transduction/tyrosine kinase

Introduction

ErbB-1/HER1 was the first transmembrane tyrosine kinase whose full primary structure was uncovered through molecular cloning (Ullrich *et al.*, 1984). Along with the isolation of additional growth factor receptors harboring an intrinsic tyrosine kinase activity, and their classification into groups of two to 15 structurally related proteins (reviewed in van der Geer *et al.*, 1994), additional members of the ErbB family were discovered. Unlike ErbB-1, which binds at least seven mammalian growth factors, whose prototype is the epidermal growth factor (EGF), no known ligand binds to ErbB-2 with high affinity. The ligands for the two other members of the family, ErbB-3 and ErbB-4, include three families of alternatively spliced growth factors, collectively called neuregulins (reviewed in Burden and Yarden, 1997). Attempts to understand the role of ErbB-2 led to the realization that this member of the family functions as a shared signaling subunit that decelerates the rate of ligand dissociation from ErbB-2-containing heterodimeric complexes (Karunagaran *et al.*, 1996). Apparently, each of the many EGF-like ligands of ErbBs acts as a bivalent molecule that binds to a primary receptor through one site of the molecule; binding of a second receptor to the other site of the ligand enables homo- or hetero-dimerization of ErbBs (Tzahar *et al.*, 1997).

Binding of an EGF-like ligand to an ErbB protein initiates a signaling cascade that culminates in recruitment of the mitogen-activated protein kinase (MAPK) pathway and results in growth or differentiation signals (reviewed in Alroy and Yarden, 1997). This pathway is conserved through evolution from worms to mammals (Kornfeld, 1997) and mutations along this pathway frequently lead to aberrant growth and malignancy. Examples include an oncogenic viral form of ErbB that induces erythroblastomas and sarcomas in birds (Downward *et al.*, 1984), a chemically induced mutant of ErbB-2 that promotes tumors in the nervous system of rodents (Bargmann *et al.*, 1986), amplification of the erbB-2 gene in several types of human adenocarcinoma (Slamon *et al.*, 1989), and autocrine production of the transforming growth factor α (TGFα), one of the ligands of ErbB-1, by virally and chemically transformed cells (reviewed in Salomon *et al.*, 1995). Significantly less is known about another autocrine loop, in which the activated growth factor genes are encoded by the invading virus, rather than by the host cell. These DNA viruses, collectively called poxviruses, are the largest of all animal viruses (reviewed in Buller and Palumbo, 1991). Poxviruses infect a wide range of species and produce remarkably different pathologies. Despite this heterogeneity, most if not all viral strains encode EGF-like growth factors that are not essential for viral replication. However, genetic inactivation experiments attributed an essential role to these secreted molecules in enhancement of virulence and stimulation of cell proliferation at the primary site of infection (McFadden *et al.*, 1996).

The EGF-like factors of only three poxviruses have been isolated: vaccinia growth factor (VGF) is synthesized after infection with the cytolytic vaccinia virus as a transmembrane precursor glycoprotein (Blomquist *et al.*, 1984; Brown *et al.*, 1985; Stroobant *et al.*, 1985). The tumorigenic viruses Myxoma virus and Shope fibroma virus encode secreted peptides, MGF and SFGF, respectively, that share 80% amino acid homology, compared with only 34–37% homology to VGF (Chang *et al.*, 1987; Upton *et al.*, 1987). Synthetic analogs of the three growth factors were synthesized and found to interact with ErbB-1,

although the affinity of interaction and the cellular outcome displayed variation and dependence on cell type (Lin *et al.*, 1988, 1990, 1991). Because these studies were performed before the ErbB family was extended to include ErbB-3 and ErbB-4, and only recently has the significance of inter-receptor interactions been fully appreciated, we hypothesized that virus-specific cytopathological landmarks, as well as cell-type specificity of infection, may be due to activation of ErbB proteins other than the EGF receptor. Here we demonstrate that each of the three viral ligands is characterized by a distinct ErbB specificity; whereas SFGF is a broad-specificity ligand that activates all ErbB-1-containing receptor combinations, in addition to the potent ErbB-2/ErbB-3 heterodimer, MGF is strictly selective to the latter receptor complex, and VGF appears to interact preferentially with ErbB-1 homodimers. Remarkably, although the viral ligands bind to the respective receptors with an affinity that is up to 1000-fold weaker than that of the relevant mammalian growth factors, their proliferative signals are similar or even higher. The underlying mechanism appears to involve attenuation of the rapid inactivation processes that are normally coupled to stimulation of signal transduction by receptor tyrosine kinases.

Results

The three viral EGF-like growth factors differ in potency and in specificity to dimeric combinations of ErbB receptors

It has been noted previously that the three known virus-encoded growth factors differ from EGF in their signaling potency and character, although they all bind to the EGF receptor (ErbB-1) (Lin *et al.*, 1988, 1990, 1991). In order to examine the hypothesis that these differences are due to interaction with ErbB proteins, other than the EGF receptor, we prepared synthetic analogs of the three viral growth factors and evaluated their ability to stimulate receptor phosphorylation in two mammary cancer cells: MDA-MB453 cells which express high levels of ErbB-2 and moderate levels of ErbB-3, but do not express ErbB-1, and MDA-MB468 cells which express high levels of ErbB-1 and moderate levels of ErbB-3, but do not express ErbB-2 (Kraus *et al.*, 1987; data not shown). For control we synthesized and similarly tested the following ligands: epiregulin (Toyoda *et al.*, 1995); EGF (47 amino acids long); and human NDF/neuregulin (a 65 amino acid-long β isoform, denoted HRGβ65; Barbacci *et al.*, 1995). Of the tested peptides, MGF and the long analog of NDF/neuregulin were most active in stimulating receptor phosphorylation in the absence of ErbB-1 (MDA-MB453 cells), but EGF and VGF were inactive (Figure 1A). In contrast, all peptides except MGF were active on cells that express no ErbB-2 (MDA-MB468 cells, Figure 1B). These results suggested that the three viral peptides differ in their specificity to ErbB proteins. This possibility was further tested on an engineered series of myeloid cell lines derived from the interleukin-3- (IL-3) dependent 32D cells (Pinkas-Kramarski *et al.*, 1996). This cellular system offers the advantage of testing ligand interaction with individual ErbB proteins, or their combinations, in the absence of endogenous ErbB proteins. Moreover, its dependence on IL-3 for survival renders this series of

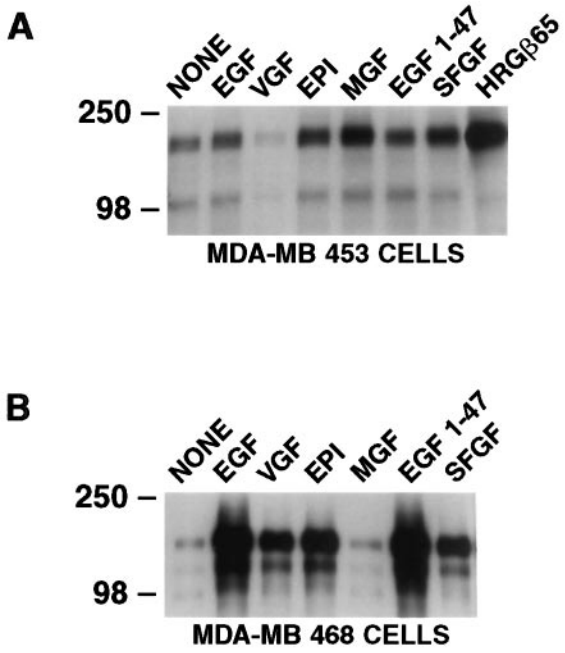

Fig. 1. Stimulation of receptor phosphorylation by EGF-like viral peptides in mammary cells. MDA-MB453 cells (**A**) and MDA-MB468 cells (**B**) grown in 48-well plates were stimulated by 50 nM (A) or 5 nM (B) of the indicated peptide for 5 min at 37°C. Total cell lysates were prepared and analyzed by immunoblotting for phosphotyrosine as described (Moyer *et al.*, 1997). All peptides used were synthetic, except for EGF (recombinant, Sigma). EPI indicates epiregulin, and HRGβ65 a 65 amino acid-long fragment of neuregulin (Barbacci *et al.*, 1995). The locations of molecular weight marker proteins are indicated in kDa. The results shown are representative of two independent experiments.

cells very sensitive to growth signals when IL-3 is omitted from the medium. Incubation of the viral growth factors with cells singly expressing ErbB-1 (D1 cells) revealed remarkable differences in the potency of the three growth factors, whereas VGF signaling was comparable to the mitogenic effect of EGF, MGF and SFGF emerged as the least and the most potent mitogens, respectively (Figure 2, D1 panel). Interestingly, not only was SFGF stimulatory at relatively low concentrations, its maximal mitogenic effect was much higher than the mitogenic action of all other growth factors we tested. Examination of cells singly expressing ErbB-2 (D2 cells) or ErbB-3 (D3 cells) revealed that no virus-encoded ligand acts as an agonist for these receptors (Figure 2; data not shown). Whereas lack of effect on ErbB-3 is likely to be due to the inactive kinase domain of this receptor (Guy *et al.*, 1994), the inability of the viral ligands to stimulate ErbB-2 is in line with the notion that this receptor acts exclusively as a shared signaling subunit of other ErbB proteins (Tzahar and Yarden, 1998). Cells singly expressing ErbB-4 (D4 cells) at a very high level responded to low concentrations of both NDF and EGF, in line with a previous report that documented binding of EGF to ErbB-4 (Shelly *et al.*, 1998), but neither viral ligand was active at concentrations <1 ng/ml (Figure 2, D4 panel). Interestingly, the activity of the ErbB-1 superagonist, namely SFGF, on ErbB-4-expressing cells was relatively low, whereas VGF emerged as the least mitogenic factor for these cells, although its effect on D1 cells was equivalent to or better than the action of EGF. Taken together, these results suggest that

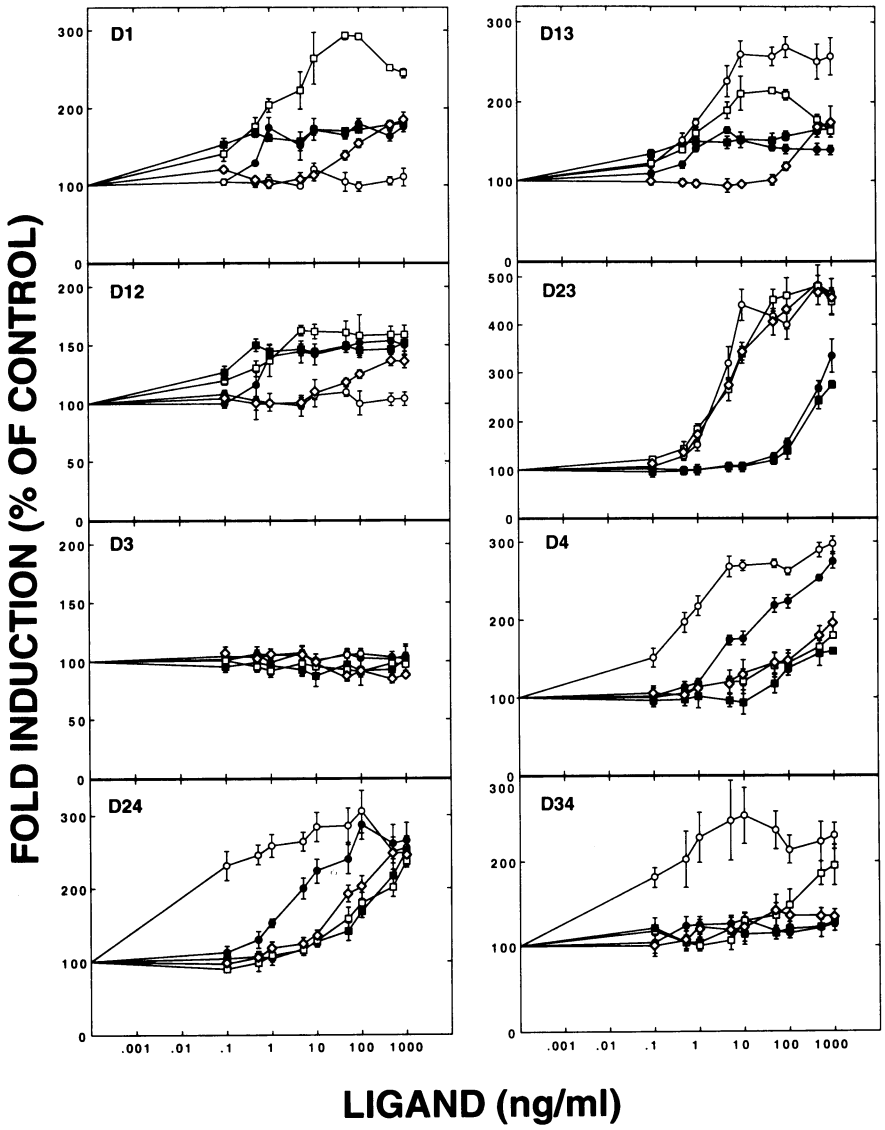

Fig. 2. Proliferative responses of ErbB-expressing derivatives of 32D cells to viral growth factors. The indicated sublines of 32D cells were tested for cell proliferation using the MTT assay. D1 cells singly express ErbB-1, whereas D12 and D13 express ErbB-1 together with ErbB-2, or with ErbB-3 (respectively). The other cell lines were named accordingly after their ErbB repertoires. Cells were plated at a density of 5×10^5 cells/ml in media deprived of IL-3 but containing serial dilutions of the following growth factors: EGF (●); NDF (○); SFGF (□); VGF (■); and MGF (◇). The MTT assay was performed 24 h after growth factor addition. The results are presented as fold induction relative to control untreated cells, and are the mean ± S.D. (bars) of four determinations. Each experiment was repeated twice with substantially similar results.

the three viral growth factors are distinct from each other, as well as from EGF, in terms of their ability to interact with ErbB-1 and ErbB-4.

Co-expression of two ErbB proteins in 32D cells permits examination of the relative signaling potency of receptor heterodimers, which are thought to be the major signaling complexes *in vivo* (Lee *et al.*, 1995; Riese *et al.*, 1995; Wallasch *et al.*, 1995; Cohen *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996). Expression of ErbB-2 together with ErbB-1 (D12 cells) elevated the basal factor-independent proliferative activity of these cells in line with previous reports (Kokai *et al.*, 1989; Cohen *et al.*, 1996; Zhang *et al.*, 1996), and, therefore, reduced sensitivity of our assay. Nevertheless, similar to the case of D1 cells, the activity of SFGF on D12 cells was higher than that of EGF and MGF, and VGF displayed better or equivalent potency to that of EGF (Figure 2, D12). This relative order of activity was reflected also in long-term survival experiments, in which D12 cells were deprived of IL-3, but their survival was prolonged, to a varying extent, by the viral growth factors (Figure 3, D12). The superior mitogenic activity of SFGF was reflected also when a combination of ErbB-1 with ErbB-3 was examined (D13 cells): although these cells responded best to NDF, the response to SFGF was greater than to EGF and VGF, and, once again, MGF emerged as the least potent mitogen of the four ligands (compare D1 with D13 in Figures 2 and 3). Surprisingly, however, MGF exerted mitogenic signals almost as potent as those of IL-3, the ultimate growth factor of 32D cells, when cells expressing a combination of the ligand-less receptor, ErbB-2, with the kinase-defective receptor, ErbB-3, were examined; on these cells, denoted D23, MGF acted not only as a potent survival factor, but it significantly stimulated cell proliferation

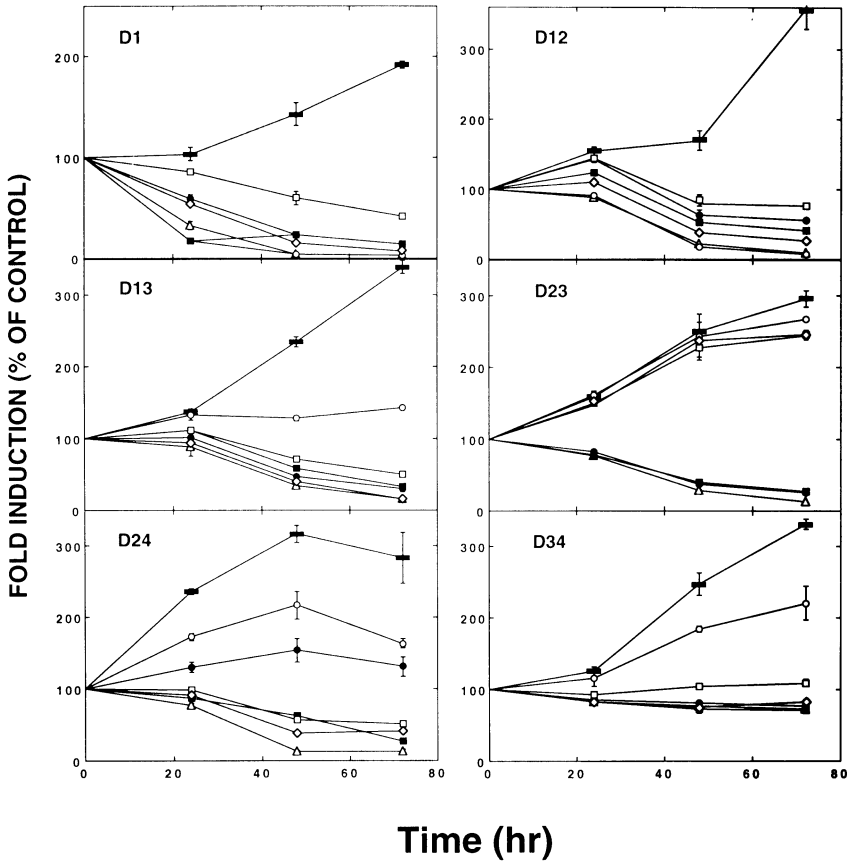

Fig. 3. Ligand-dependent survival of ErbB-expressing cells. The indicated sublines of 32D cells were incubated at a density of 5×10^5 cells/ml with media supplied with IL-3 (■), or with the following growth factors (each at 100 ng/ml): EGF (●); NDF (○); VGF (■); SFGF (□); and MGF (◇). For control, we incubated cells with no factor (open triangles). The extent of cell proliferation was determined daily by using the colorimetric MTT assay. The data presented are the mean ± S.D. of four determinations. The experiment was repeated twice with similar results.

(D23 in Figures 2 and 3). Likewise, SFGF and NDF displayed potent actions on D23 cells, but EGF and VGF were active only at very high concentrations (>200 ng/ml), in line with two recent reports (Alimandi *et al.*, 1997; Pinkas-Kramarski *et al.*, 1998). It is interesting to note that no other combination of ErbB-2 or ErbB-3 with other receptors was able to potentiate the effect of MGF to the level observed with D23 cells, although this factor was slightly more potent than SFGF on D24 cells, and only very weakly stimulated D34 cells (D24 and D34 in Figures 2 and 3). When compared with either NDF or EGF, MGF emerged as a weak mitogen for all other receptor combinations, including ErbB-1/ErbB-2 and ErbB-1/ErbB-3. In conclusion, our results indicate that the three viral growth factors can stimulate ErbB proteins other than the EGF receptor (ErbB-1), but they differ significantly from one another and from EGF: VGF is as potent as EGF on ErbB-1-containing combinations, but is much weaker than EGF on homo- and hetero-dimers of ErbB-4. On the other hand, SFGF emerges as a superagonist of ErbB-1-containing receptor combinations, but like VGF its ability to stimulate homo- or hetero-dimeric complexes of ErbB-4 is rather weak. The third viral ligand, MGF, exhibits the most narrow selectivity; its action is practically limited to cells co-expressing a combination of ErbB-2 with ErbB-3.

The convergence of signal transduction by all ErbB combinations at the mitogen-activated protein kinase (MAPK) pathway (Alroy and Yarden, 1997) enabled us to support the results obtained using cell proliferation or survival assays. The various derivatives of 32D cells were incubated for 10 min with a relatively high concentration of each viral ligand, and the state of receptor activation determined by using antibodies to phosphotyrosine. To assay MAPK activation we used a monoclonal antibody that specifically recognizes the active, doubly phosphorylated form of the ERK1 and ERK2 MAPKs (Yung *et al.*, 1997). The results of these analyses are shown in Figure 4. Evidently, ErbB-1 phosphorylation by EGF was greater than the effect of the three viral ligands on cells singly expressing this receptor (D1 cells). In fact, a hyperphosphorylated form of ErbB-1, whose electrophoretic mobility is slower, was most prominent in EGF-treated cells. A similar picture emerged when cells expressing a combination of ErbB-1 with ErbB-3 (D13 cells) were examined. This contrasted with the observation that the mitogenic responses of SFGF and VGF were higher than that of EGF on all ErbB-1-expressing cells we examined (Figures 2 and 3). A better reflection of the relative mitogenic potency was provided by the analysis of MAPK: activation by SFGF, as well as by VGF, was comparable to that observed with EGF, whereas the least mitogenic ligand, MGF, induced only weak signals in D1 and in D13 cells. As expected, however, MGF potently stimulated receptor phosphorylation, as well as MAPK activation, in cells co-expressing ErbB-2 and ErbB-3 (D23 in Figure 4), in

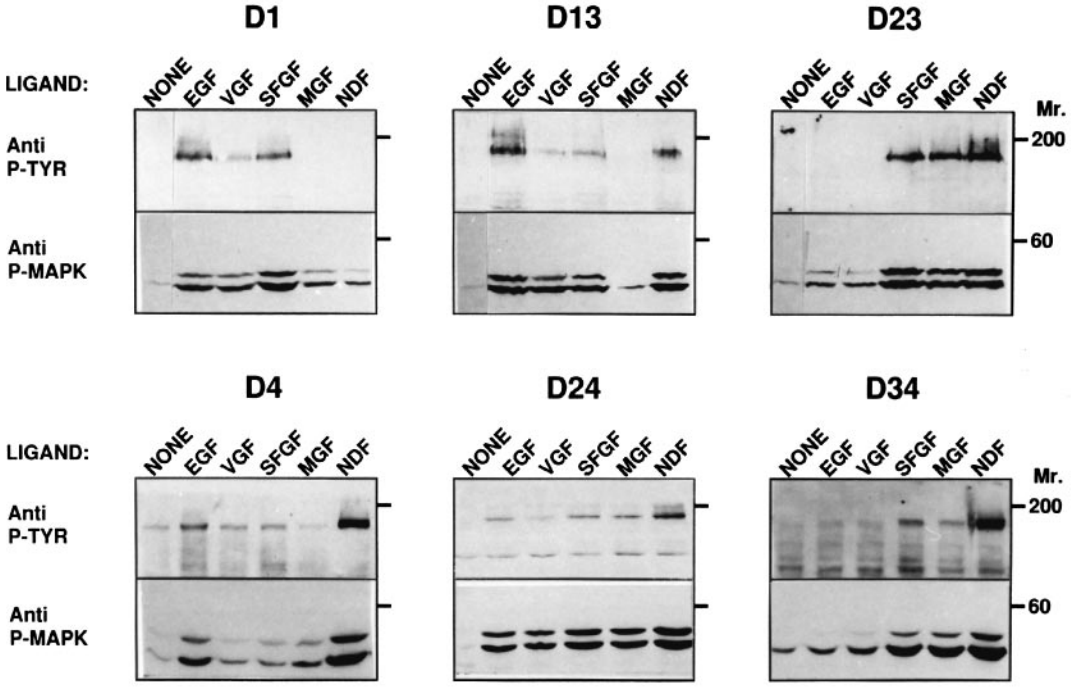

Fig. 4. Receptor phosphorylation and MAPK activation in response to viral ligands. The indicated cell-lines were incubated for 10 min at 22°C with various peptide growth factors (each at 100 ng/ml). Control cultures were incubated with no added factor (NONE). The reaction was terminated by adding hot (95°C) gel sample buffer. The resultant whole cell lysates were subjected to gel electrophoresis, followed by immunoblot analysis with either an anti-phosphotyrosine antibody (PY-20; upper panels), or with antibodies directed to the activated, double phosphorylated form of MAPK (P-MAPK; lower panels). Signals were developed using an ECL kit (Amersham). Note that D4 cells express ~10-fold more ErbB-4 molecules than the D24 and D34 cell lines.

agreement with the potent mitogenic signal observed with D23 cells (D23 in Figures 2 and 3), and the effect of this ligand on mammary cells (Figure 1). Likewise, NDF and SFGF, which acted as potent mitogens on D23 cells, were as active as MGF in the MAPK assay, but the non-mitogenic ligands of D23 cells, namely EGF and VGF, exerted no effect on MAPK. Similarly, analyses of ErbB-4-expressing cells revealed some concordance between MAPK activation and the mitogenic effect of the growth factors we tested. Thus, NDF exerted the most potent effects on both ErbB phosphorylation and MAPK activation in D4, D24 and D34 cells, and the second potent ligand for D4 and D24 cells, namely EGF, induced slightly lower signals (Figure 4). The effects of SFGF and MGF on receptor phosphorylation in ErbB-4-expressing cells were weak compared with NDF, although their ability to stimulate MAPK in these cells was surprisingly high. This raised the possibility that the kinetics, rather than potency, of MAPK activation by the viral ligands is critical for growth signals (see below). In conclusion, receptor phosphorylation and MAPK activation by the viral ligands only partially correlate with the longer lasting growth effects.

Ligand displacement analyses reveal discordance between signaling potency and binding affinities of viral ligands

The large differences in signaling potency that we observed with the three viral ligands, and especially the difference between SFGF and EGF on ErbB-1-expressing cells (D1 in Figure 2), raised the possibility that ligand-binding affinities may explain how the same receptor combination can generate weak or strong signals in response to ligand binding. Two series of cell lines were used to test this scenario by using ligand-displacement analyses. In addition to the 32D cell derivatives described above, we employed a similar set of Chinese hamster ovary (CHO) cells that express various combinations of the ErbB proteins on a low background of an endogenous hamster ErbB-2 (Tzahar *et al.*, 1996). Due to their adhesion to the substrate, the CHO-derived cells (denoted CB cells), unlike the non-adherent 32D cells, enable more sensitive ligand-binding assays. Competition between increasing concentrations of an unlabeled EGF and a relatively low concentration of ^{125}I-EGF showed that the apparent affinity of this ligand to 32D or CHO cells singly expressing ErbB-1 (D1 and CB1 cells, respectively) was in the range of 0.2–1 nM, but the apparent affinity of VGF was 5- to 7-fold lower (CB1 and D1 panels in Figure 5). Surprisingly, the other two viral ligands, SFGF and MGF, displayed much lower binding affinities: on the basis of the results obtained with CB1 cells we estimate that the affinity of SFGF is 100- to 1000-fold lower than that of EGF, and MGF displayed an even less tight binding to ErbB-1 (CB1 in Figure 5). Similar displacement analyses that used ^{125}I-NDF as a tracer revealed very large differences between the apparent binding affinities of SFGF, MGF and NDF towards cells co-expressing ErbB-2 together with ErbB-3 (CB23 and D23 in Figure 5), although the three ligands were almost equipotent in cell proliferation assays (D23 in Figures 2 and 3). Remarkably, the affinity of MGF was slightly better than that of SFGF, but the affinities of both ligands were 2–3 orders of magnitude lower than the apparent affinity of NDF. Thus, similar to the discrepancy

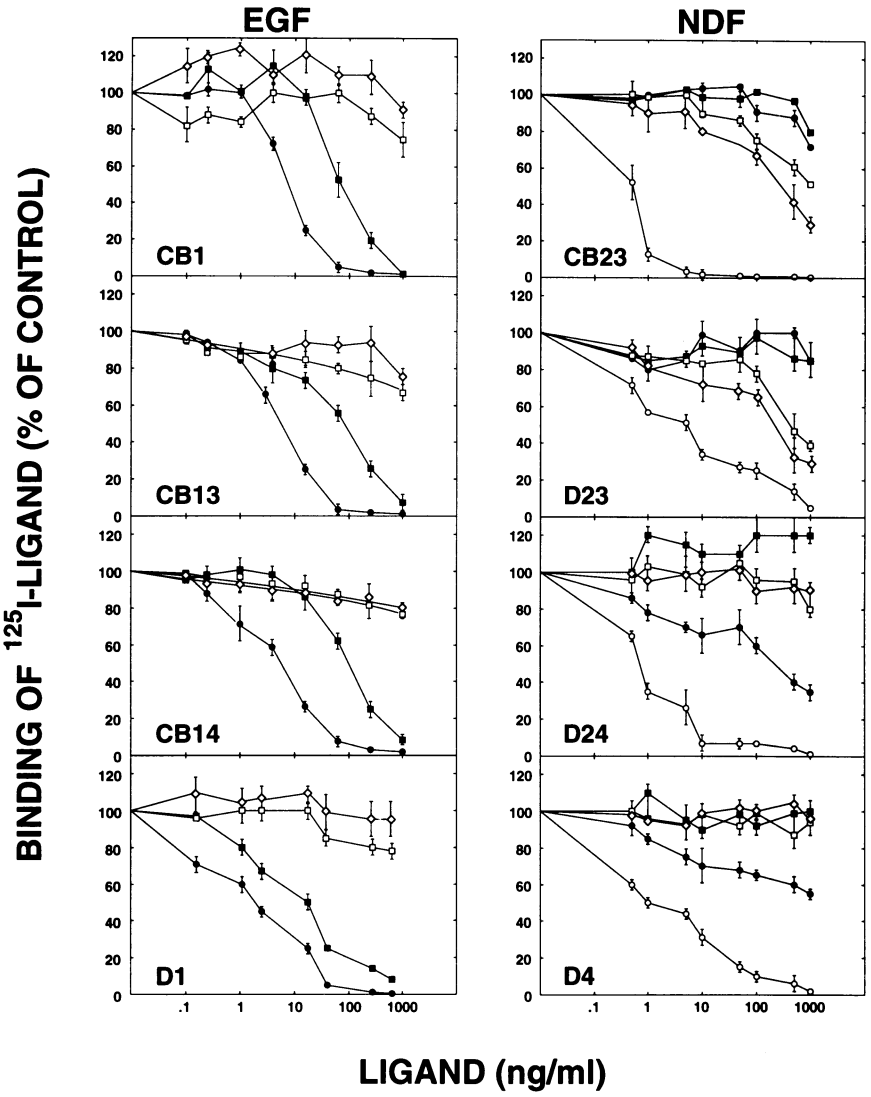

Fig. 5. Displacement of radiolabeled EGF or NDF molecules by viral growth factors. Two engineered series of ErbB-expressing cell lines were used for radioligand displacement analysis. The D cell lines are derivatives of 32D myeloid cells, whereas the CB cell lines were derived from CHO cells by transfecting the respective erbB cDNAs. For example, CB23 cells co-express ErbB-2 and ErbB-3. Cells were incubated for 2 h at 4°C with 1 ng/ml of either ^{125}I-EGF (left column) or ^{125}I-NDF (right column), in the presence of the indicated increasing concentrations of unlabeled EGF (●), NDF (○), VGF (■), SFGF (□) or MGF (◇). Unbound ligands were removed as described under Materials and methods, and cell-associated radioactivity determined by counting gamma irradiation. Each data point represents the mean ± range (bars) of two determinations. The experiment was repeated twice.

observed in ErbB-1-expressing cells, both SFGF and MGF stimulated D23 cells far better than would be expected on the basis of their relative binding affinities. This discrepancy is in marked contrast with the non-viral ligands we tested, namely EGF and NDF, whose mitogenic potencies well reflected binding affinities. For example, in line with the 10- to 100-fold lower mitogenic potency of EGF relative to NDF, for cells expressing ErbB-4 (either alone or in combination with ErbB-2; Figure 2), this ligand displayed a correspondingly lower binding affinity to erbB-4-expressing cells (D4 and D24 in Figure 5). Once again, despite extremely low affinity of the three viral ligands to ErbB-4 expressing cells (Figure 5), these ligands were nevertheless mitogenic for D4 cells, although at concentrations >10 ng/ml (Figure 2).

Our attempts to determine binding parameters of the three viral ligands by directly using their radiolabeled forms have failed (data not shown), probably due to the very low affinity of these ligands to all combinations of ErbB proteins. However, blocking ligand dissociation, by using a covalent crosslinking reagent, provided us with a qualitative binding assay, and also permitted examination of the ability of the viral ligands to induce various homo- and hetero-dimers of ErbBs. The results of these affinity-labeling experiments are presented in Figure 6. Evidently, all three ligands labeled monomers and dimers of ErbB-1, as well as both species of ErbB-4, at variable efficiency. Although quantitative comparison of the efficiency of receptor labeling by the three ligands may not reliably reflect their relative binding affinities, due to potential sequence-specific differences in the extent of labeling and chemistry of crosslinking, the ratio of dimers to monomers, and especially the extent of co-immunoprecipitation of the affinity-labeled ErbBs, may be used as

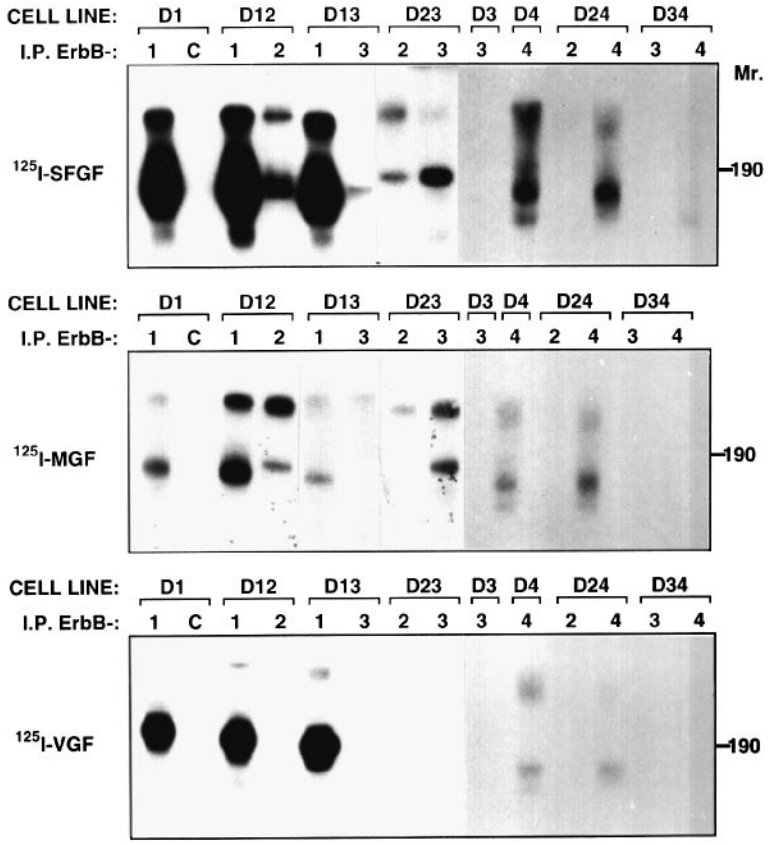

Fig. 6. Covalent crosslinking of radiolabeled viral ligands to 32D cell derivatives expressing individual ErbBs and their combinations. The indicated derivatives of 32D cells (0.5×10^7 per lane) were separately incubated with the three radiolabeled viral peptides (250 ng/ml), as indicated. Following 2 h at 4°C, the covalent crosslinking reagent *bis(*sulfosuccinimdyl)-suberate (BS^3) was added (1 mM) for an additional incubation for 45 min. Then cell lysates were prepared and subjected to immunoprecipitation with antibodies against the indicated ErbB proteins, or with a control antibody, labeled C. The complexes were resolved by gel electrophoresis, followed by autoradiography. Exposure times of the X-ray films were 72, 48 and 36 h, for SFGF, MGF and VGF, respectively. The locations of molecular weight marker proteins are indicated in kDa.

an indicator of ligand-induced receptor interactions. For example, ErbB-2 was best recruited by MGF into heterodimers with ErbB-1 (D12 lanes in Figure 6). It is important to note that no ligand interacted detectably with ErbB-3 when this receptor was present alone (D3 lanes, Figure 6). However, ErbB-3 was quite efficiently labeled by both SFGF and MGF, but not by VGF, when co-expressed with ErbB-2 (D23 lanes in Figure 6). Remarkably, in D23 cells MGF promoted homodimers of ErbB-3, and heterodimers between ErbB-2 and ErbB-3, more efficiently than did SFGF (D23 lanes in Figure 6). Taken together, the superior ability of MGF to engage heterodimers of ErbB-2 with either ErbB-3 or with ErbB-1 implies that this growth factor evolved as a heterodimer-specific ligand. This may explain why MGF displayed very low binding and mitogenicity when tested on cells singly expressing ErbB-1, as opposed to its high activity on the combination ErbB-2/ErbB-3 (D23 in Figures 2 and 3, and CB23 and D23 in Figure 5). By contrast with the heterodimer specificity of MGF, VGF emerged as a ligand that hardly forms heterodimers: practically, only monomeric forms of ErbB-1 were observed in D1, D12 and D13 cells labeled with ^{125}I-VGF. However, although VGF bound very weakly to ErbB-4, it detectably engaged dimers of this receptor (Figure 6), but this led to only low mitogenicity (Figure 2).

SFGF and EGF signal through similar pathways but the viral ligand induces sustained MAPK activation in the nucleus

We next addressed the mechanism that confers signaling superiority to SFGF despite the extremely low affinity of this ligand to ErbB receptors. Two models were examined: according to the first, the viral ligands bind to a site(s) of ErbB-1 that is distinct from the EGF-binding cleft, and therefore these ligands differ in their potency of receptor stimulation. However, covalent crosslinking experiments with D1 cells indicated that a monoclonal antibody (mAb) that selectively binds to the EGF-binding cleft of ErbB-1 (mAb111) inhibited binding of SFGF, as well as that of EGF, to ErbB-1 (Figure 7A). A second ErbB-1-specific antibody, mAb199, affected the binding of neither EGF nor SFGF, implying that the two ligands bind to immunologically indistinguishable sites of ErbB-1. This conclusion was confirmed by using three additional mAbs to the human ErbB-1 (data not shown). According to the second model the viral and the mammalian ligands similarly induce receptor activation, but after binding of SFGF, ErbB-1 couples to a set of signaling proteins that is distinct from the collection of cytoplasmic effectors that are recruited by an EGF-bound ErbB-1. Several downstream effectors of ErbB-1 were examined using immunoblot analysis of whole lysates derived from ligand-stimulated D1 cells. Two examples

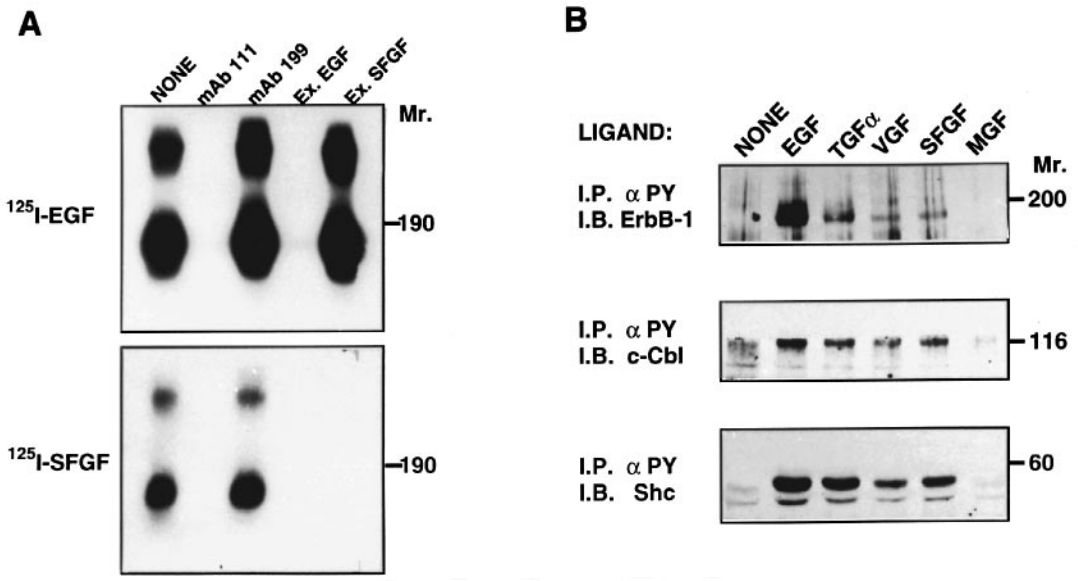

Fig. 7. Comparison of signaling by viral and mammalian ligands. (**A**) Effect of mAbs on binding of SFGF and EGF to ErbB-1. Covalent crosslinking of radiolabeled EGF (upper panel) or a radiolabeled SFGF (lower panel) to D1 cells was performed as described in the legend to Figure 6, except that incubation with the radiolabeled ligands was performed in the presence of one of the following mAbs (each at 20 μg/ml): mAb111 that inhibits EGF binding to ErbB-1, and mAb199 that does not affect the binding of EGF to ErbB-1. For control, cells were incubated in the absence of either mAb (lanes labeled NONE), or in the presence of a 100-fold higher concentration of the indicated unlabeled ligands (Ex. EGF or Ex. SFGF). Following covalent crosslinking with BS^3 and preparation of whole cell lysates, ErbB-1 was subjected to immunoprecipitation using a rabbit antiserum directed against a synthetic peptide derived from the C-terminus of ErbB-1. Note that the specific activities of the two radiolabeled ligands were comparable, but nevertheless labeling with EGF was more intense. (**B**) Recruitment of cytoplasmic signaling proteins by ErbB-1 in response to ligand stimulation. D1 cells (0.1×10^6 cells/lane) singly expressing ErbB-1 on the cellular background of the 32D cell line were incubated for 10 min at 37°C with the indicated ligands, each at 60 ng/ml. Control cultures were incubated with no added factor (NONE). Whole cell lysates were then prepared and subjected to immunoprecipitation (I.P.) with an anti-phosphotyrosine mAb. The resulting protein blots were subjected to an immunoblot analysis (I.B.) with antibodies to ErbB-1, c-Cbl, or Shc, as indicated.

are shown in Figure 7B: the c-Cbl adaptor protein, whose engagement is stimulated by ErbB-1, but not by other ErbB proteins (Levkowitz *et al.*, 1996), and Shc, a common effector of all ErbB proteins (Culouscou *et al.*, 1995). Evidently, both protein transducers underwent tyrosine phosphorylation in response to the viral ligands SFGF and VGF, but MGF hardly affected them, in accordance with the very weak mitogenic effect of this ligand on cells singly expressing ErbB-1 (Figure 2). Two well-characterized mammalian ErbB-1 ligands, EGF and TGFα, led to comparable phosphorylation of c-Cbl and Shc, although receptor activation by these ligands was significantly higher than the action of the three viral ligands (Figure 7B, upper panel). Activation of MAPK, c-Jun N-terminal kinase and p38RK (HOG; reviewed in Davis, 1994), along with several other cytoplasmic proteins, was another feature shared by SFGF, VGF, EGF and TGFα (data not shown), suggesting that overlapping, if not identical, sets of signaling proteins are recruited by all ErbB-1 ligands.

Because the signaling pathways activated by SFGF and EGF appear similar, and on the other hand signaling by all ErbB proteins is funneled into the MAPK pathway (reviewed in Alroy and Yarden, 1997), we considered the possibility that SFGF signaling is unexpectedly potent due to differences in the kinetics of MAPK activation. To test this prediction we stimulated D1 cells with either SFGF or EGF and examined the state of MAPK activation at increasing time intervals. Remarkable differences in the kinetics of MAPK activation were uncovered by this experiment (Figure 8A): whereas EGF induced transient activation of MAPK that peaked at 2–5 min, the enzyme remained in its active state 1 h after stimulation with SFGF. By contrast, tyrosine phosphorylation of ErbB-1 was much stronger after binding of EGF, but in both cases receptor stimulation reached a peak at ~10 min and then declined slowly. We concluded that the relatively weak binding of SFGF to ErbB-1 is followed by a limited stimulation of tyrosine phosphorylation of this receptor, but this is sufficient for sustained and quite potent activation of the MAPK pathway.

It has been shown previously that MAPK activation is followed by rapid translocation of the phosphorylated kinase to the nucleus (Chen *et al.*, 1992; Lenormand *et al.*, 1993), where it stimulates transcription of specific genes. To test the prediction that SFGF more efficiently translocates MAPK to the nucleus than does the mammalian growth factor, we followed the subcellular localization of MAPK by using antibodies specific to the active form of the kinase. Whereas in unstimulated D1 cells only background cellular staining was observed, binding of either EGF or SFGF was followed by rapid appearance of the active form of MAPK in the nucleus (Figure 8B; staining with a DNA-intercalating dye, DAPI, allowed visualization of nuclei). Consistent with the results shown in Figure 8A, immunostaining was more prominent in cells stimulated with SFGF and it lasted for longer.

Receptor ubiquitination and inactivation in response to viral growth factors is attenuated

The observed sustained MAPK activation by SFGF, in combination with the relatively weak receptor phosphorylation that was induced by this ligand, hinted that the process of signal termination, rather than signal activation,

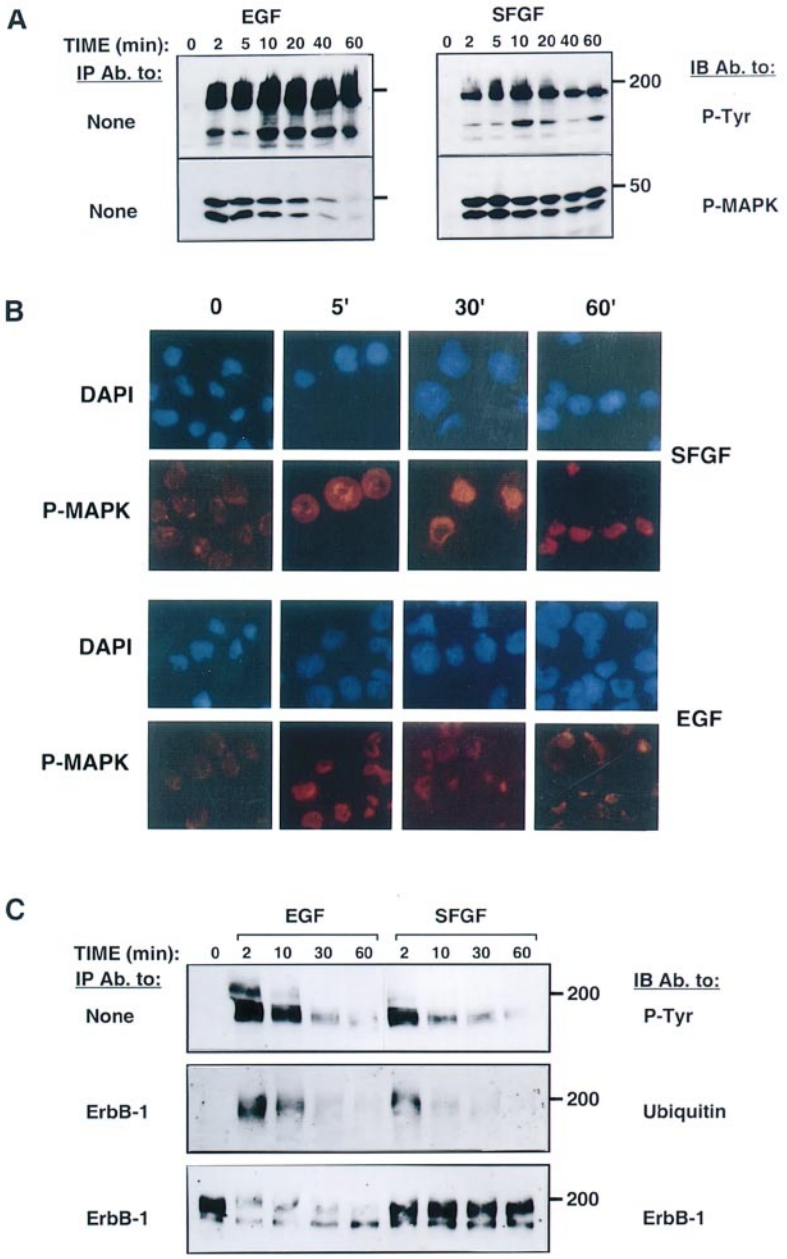

Fig. 8. Time course of ligand-mediated receptor phosphorylation, MAPK activation and retention in the nucleus, and receptor ubiquitination/degradation. (**A**) D1 cells (0.1×10^6 cells/lane) were incubated at 37°C with either SFGF or EGF (each at 50 ng/ml). Following the indicated time intervals, cell stimulation was terminated by adding boiling sample buffer directly on cells. Whole cell lysates were then subjected to immunoblot analysis with either an anti-phosphotyrosine mAb (upper panels), or a mAb directed to the active, doubly phosphorylated form of MAPK (lower panels). The locations of molecular weight marker proteins are indicated in kDa. (**B**) D1 cells (5×10^4) were stimulated at 37°C with EGF or SFGF for the indicated periods of time. Cytospin preparations of these cultures were then fixed and cells permeabilized by incubation in PBS containing Triton X-100 (0.1%). To visualize nuclear DNA, cells were stained with DAPI. Active MAPK was detected by using a mAb specific to the active doubly phosphorylated form. (**C**) D1 cells (10^7 cells/lane) were stimulated at 37°C with EGF or SFGF (50 ng/ml each). Whole cell extracts were prepared at the end of the indicated periods of time and divided into two aliquots. One fraction (20%) was directly subjected to gel electrophoresis, while the other aliquot was subjected to immunoprecipitation (IP) with antibodies to ErbB-1. Gel electrophoresis (7.5% polyacrylamide) of cell lysates or immunoprecipitates was followed by immunoblotting (IB) with antibodies to either phosphotyrosine (P-Tyr) or ubiquitin. The anti-ubiquitin blot was used for re-blotting with a mAb directed to the C-terminal peptide of ErbB-1. Note that alignment of the two lower panels identified the ubiquitinated protein band as the minor upper band of the tyrosine-phosphorylated ErbB-1.

is important for understanding how SFGF potentiates mitogenic signals. Perhaps the most prominent mechanism of receptor inactivation, termed downregulation, is the rapid removal of activated ligand–receptor complexes from the cell surface, and their proteolysis in intracellular vesicular compartments (reviewed in Sorkin and Waters, 1993). We first compared the abilities of SFGF and EGF to induce degradation of ErbB-1 in D1 cells. Immunoblot analysis of ErbB-1 isolated from ligand-stimulated cells revealed rapid disappearance of the 170 kDa receptor band upon short exposure to EGF (Figure 8C, lower panel). In contrast, SFGF induced only limited receptor degradation, even after long exposure to the viral ligand (Figure 8C, lower panel). Because ErbB-1 degradation

involves elevated ubiquitination, and both ubiquitination and degradation depend on endocytosis of the ligand–receptor complexes (Galcheva-Gargova *et al.*, 1995), we compared the state of receptor ubiquitination by using two methods: electrophoretic resolution of the ubiquitinated species, whose electrophoretic mobility is retarded (Figure 8C, upper panel), and direct identification of receptor–ubiquitin complexes by using anti-ubiquitin antibodies (Figure 8C, middle panel). These analyses indicated that EGF causes rapid appearance of a high molecular weight tyrosine phosphorylated band, which was recognized by anti-ubiquitin antibodies. The appearance of this minor phosphorylated band was significantly lower after cell stimulation by SFGF, and the induction of receptor ubiquitination by this viral ligand was limited and transient compared with that induced by the mammalian ligand. Taken together, the results presented in Figure 8C indicated that receptor ubiquitination and degradation were less efficiently induced by SFGF.

To follow downregulation of ErbB-1 we chose CB1 cells because these adherent cells, unlike 32D derivatives, offer a more sensitive experimental system, yet ligand endocytosis and degradation, as well as sustained MAPK activation, are comparable to the processes exhibited by D1 cells (Pinkas-Kramarski *et al.*, 1996; Tzahar *et al.*, 1996; data not shown). CB1 cells were first incubated at 37°C with the three viral ligands, or with a relatively high concentration of EGF, and the level of ErbB-1 that remained at the cell surface was then determined by performing a binding assay with a radiolabeled derivative of EGF. The results of this experiment revealed that whereas most (>70%) surface-exposed ErbB-1 molecules disappeared after a 3 h incubation with EGF, only a 10–25% reduction was induced by MGF or SFGF (Figure 9A). Consistent with the moderate binding affinity of VGF, and the relatively high mitogenic activity of this ligand, it induced only partial downregulation of ErbB-1 (Figure 9A). This pattern of relative receptor downregulation was independent of ligand concentration (Figure 9B). Moreover, a qualitative difference between a viral ligand (SFGF) and the mammalian growth factor was confirmed by performing the following experiment: dose–response assays of ligand-induced receptor phosphorylation were carried out and equipotent concentrations selected (e.g. 62 ng/ml SFGF and 1.2 ng/ml EGF). At these concentrations the difference in potency of receptor downregulation was retained (data not shown), in line with dissimilar mechanisms.

The cellular fate of ErbB-1 after binding of viral ligands was followed by an alternative approach that used immunolocalization and fluorescence microscopy (Figure 9C). In the absence of a ligand, ErbB-1 was localized primarily to the cell surface where it formed either small patches or a uniform pattern. Within 2–5 min after binding of EGF, most ErbB-1 molecules underwent internalization and localized to vesicular structures representing endosomes. Later (15–20 min), these structures assumed a perinuclear localization and their size increased, probably reflecting vesicle fusion and arrival at lysosomal or prelysosomal compartments (data not shown). By contrast with an EGF-driven ErbB-1, only limited redistribution of the receptor took place even after long incubation with SFGF (Figure 9C; data not shown). In addition to the membrane localization of ErbB-1 molecules in SFGF-treated cells, the receptors formed small patches that were either at the cell surface, or very close underneath the plasma membrane. Thus, on the basis of both biochemical and structural lines of evidence it seems that the viral ligand, SFGF, is unable to direct ErbB-1 to large endocytic vesicles and, thereby, to intracellular degradation. Conceivably, the altered routing of ErbB-1 prolongs the active state of the ligand–receptor complex, thus augmenting the mitogenic signal of the viral growth factor.

Discussion

Investigation of animal viruses has provided many insights into basic molecular mechanisms. Perhaps the best example is the lessons in cellular transformation and cell-cycle control that evolved from studies of RNA-containing retroviruses and papovaviruses (e.g. SV-40). Likewise, poxviruses, whose most notable member is the causative agent of smallpox in humans, the variola virus, emerge as a rich source of efficient mechanisms to evade the immune system. For example, the Myxoma T2 protein is a homolog of the tumor necrosis factor- (TNF) receptor that blocks TNF-mediated cytotoxicity (Smith *et al.*, 1990), and vaccinia virus encodes a complement-binding protein that blocks the classical complement pathway (Kotwal and Moss, 1988). Unlike these two proteins that are involved in secondary infection via the efferent lymphatics and blood stream, virus-encoded growth factors play a critical role at the major portal of viral entry, the skin; by induction of localized hyperplasia, additional metabolically active cells become available for viral infection (reviewed in Buller and Palumbo, 1991). However, in contrast to the current notion, our results imply that the viral growth factors do not function simply as alternative agonists of the EGF receptor. Instead, these molecules utilize two novel features: first, each viral ligand is characterized by a unique pattern of specificity to homo- or hetero-dimeric complexes of the ErbB receptors. Secondly, the viral growth factors are biologically far more potent than their mammalian counterparts in terms of receptor occupancy. The underlying molecular mechanisms and their implications for poxvirus pathogenesis are discussed below.

Narrow versus broad ErbB specificity of viral growth factors

Collectively, analyses of cellular proliferation (Figure 2), or survival (Figure 3), ligand displacement (Figure 5), and covalent crosslinking (Figure 6), revealed a ligand-specific pattern of receptor specificity that is schematically presented in Figure 10. This pattern implies that the three viral ligands are functionally distinct. Yet, the mammalian ligand that we used as a reference, namely EGF, differs from the three viral ligands. Thus, whereas EGF can activate all ErbB-1-containing complexes, as well as homodimers of ErbB-4 (when this receptor is overexpressed; Shelly *et al.*, 1998) and ErbB-2/ErbB-4 heterodimers, SFGF differs in that only at very high concentrations (>50 ng/ml) this ligand detectably activates ErbB-4, but, on the other hand, it can efficiently stimulate the ErbB-2/ErbB-3 heterodimer. By contrast, stimulation of the latter by EGF occurs only at very high concentrations (Alimandi

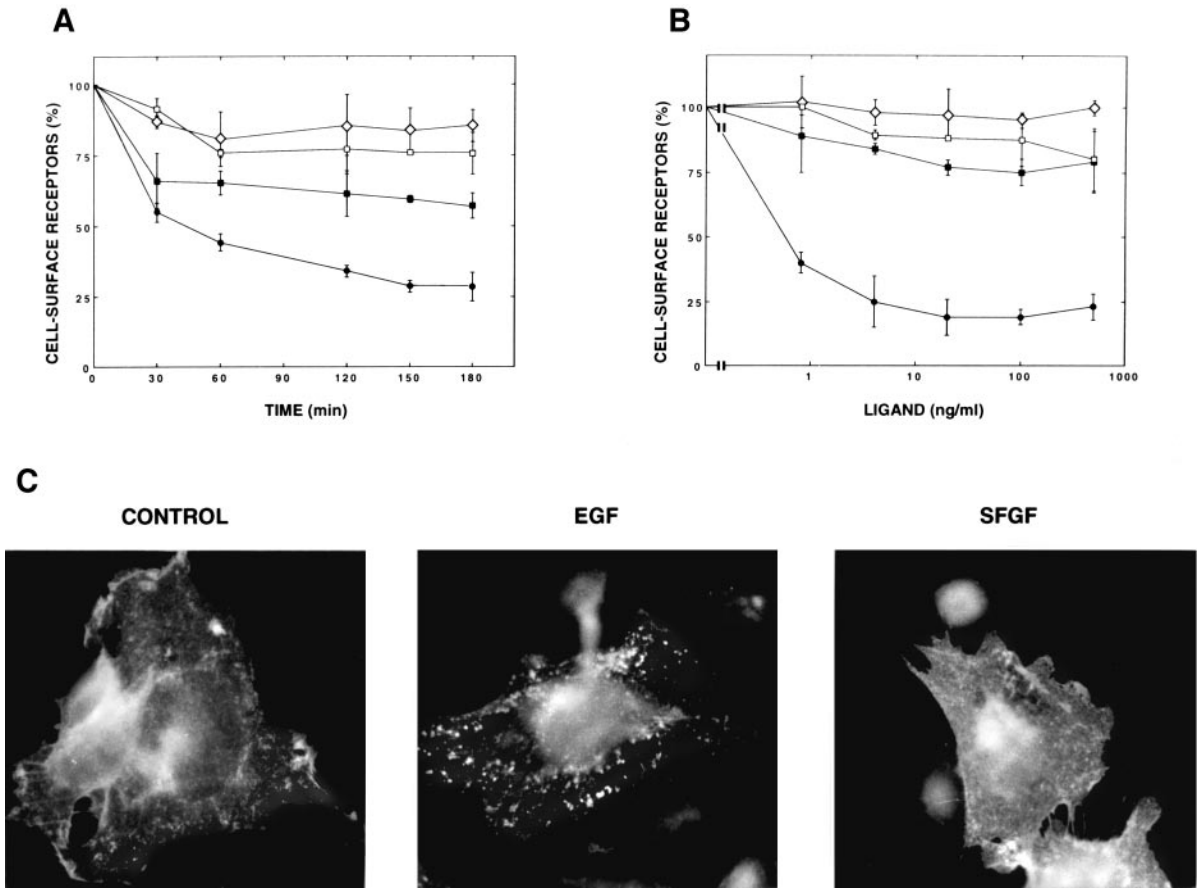

Fig. 9. Intracellular trafficking of ErbB-1 in response to stimulation with SFGF or EGF. CHO cells singly expressing ErbB-1 (denoted CB1 cells) on a low background of the endogenous hamster ErbB-2 were used in all panels. (A and B) Downregulation of ErbB-1 was followed by incubating subconfluent monolayers of CB1 cells (250 000 cells in 24-well trays) with one of the following unlabeled ligands: EGF (●); SFGF (□); VGF (■); and MGF (◇). Cells were treated at 37°C, either for various time intervals with 60 ng/ml ligand (**A**), or with various ligand concentrations for 2 h (**B**). Cell surface-bound ligands were removed by acid wash, which was followed by extensive rinsing with binding buffer. The receptor level was then determined by binding of radiolabeled EGF (5 ng/ml) to the cells (2 h at 4°C). (**C**) For fluorescence labeling of ligand-stimulated ErbB-1, CB1 cells were treated for 5 min at 37°C with EGF or with SFGF, each at 250 ng/ml, as indicated. Control monolayers were treated with buffer alone (CONTROL). Cells were then washed, fixed, permeabilized and stained with antibodies against ErbB-1 (mAb111) as described in Materials and methods. The coverslips were viewed with a Zeiss fluorescence microscope with an oil immersion objective.

et al., 1997; Pinkas-Kramarski *et al.*, 1998). Nevertheless, this property is shared by betacellulin, but not by TGFα, which led to the conclusion that ErbB-1-specific ligands differ in their ability to stimulate the ErbB-2/ErbB-3 heterodimer (Alimandi *et al.*, 1997; Pinkas-Kramarski *et al.*, 1998). This conclusion is in line with the emerging notion (Tzahar and Yarden, 1998) that the multiple EGF-like ligands are distinct in terms of receptor specificity (Beerli and Hynes, 1996; Riese *et al.*, 1996). A remarkable demonstration is provided by MGF; the exclusive receptor of this ligand is the ErbB-2/ErbB-3 heterodimer. It is notable that no other known EGF-like ligand exhibits such a narrow specificity, and neither ErbB-2 nor ErbB-3, when singly expressed, detectably interacts with MGF (data not shown). An intermediate case is provided by VGF: this growth factor binds to and activates cells expressing ErbB-1 in various combinations, but covalent crosslinking analyses of this ligand suggest that it hardly forms heterodimeric complexes of ErbB-1 at all (Figure 6).

Because previous analyses examined the effects of viral ligands on naturally occurring cell lines (e.g. the epidermoid carcinoma A-431 cells) expressing various combinations of ErbBs, it is difficult to compare our results with previous works. However, it has been reported previously that the cell-binding affinity of MGF is 200-fold lower than that of EGF (Lin *et al.*, 1991), an observation that is consistent with our results (Figure 5). Likewise, synthetic analogs of VGF (Lin *et al.*, 1990) and SFGF (Lin *et al.*, 1988) were found to be 5- to 10-fold less potent than EGF in binding to A-431 cells. Our results with VGF are in line with this observation, but we noted a much higher difference with SFGF (Figure 5). Another discrepancy is related to the biological activity: whereas according to our results SFGF and VGF are mitogenically superior or equivalent to EGF on all ErbB-1-expressing 32D cell derivatives (Figure 2), the previously reported mitogenic effect of SFGF on NRK cells was 10-fold lower than that of EGF (Lin *et al.*, 1988), and VGF was either inactive or antagonistic to EGF (Lin *et al.*, 1990). These differences may, in part, be attributed to the presence of ErbB-4, or to other cell type-specific features. In line with this possibility, we observed an inhibitory effect of VGF on ErbB-1 phosphorylation in epithelial cells, but not in engineered myeloid cells (Figures 1 and 4).

Which selective advantages could poxviruses gain from fine targeting of their growth factors? The mitogenic effect

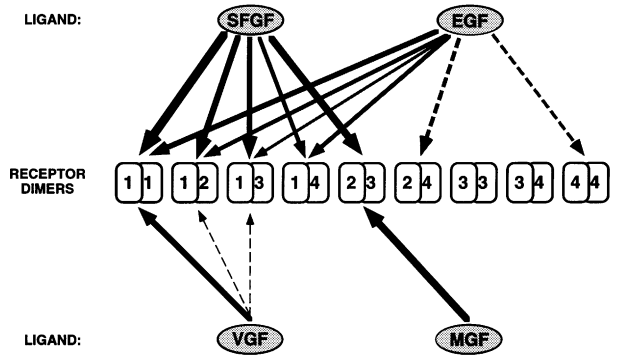

Fig. 10. Receptor specificity of viral growth factors. All possible combinations of ErbB proteins are presented schematically, except for the ErbB-2 homodimer. Specific ErbB proteins are identified by their numbers. The interaction between a growth factor and a specific dimer of ErbBs was inferred on the basis of the following criteria: the ability to stimulate growth of IL-3-dependent cells (Figures 2 and 3); activation of MAPK and receptor phosphorylation (Figure 4); binding to cells expressing specific ErbBs (Figure 5); and covalent crosslinking to specific monomeric and dimeric receptor complexes (Figure 6). These interactions are shown by arrows whose thicknesses represent the relative potency of biological effects (broken arrows indicate weak receptor activation). Interactions that are detectable only at high ligand concentrations (>10 ng/ml) are not represented. For example, the low-affinity interaction between EGF and the ErbB-2/ErbB-3 heterodimer (Alimandi *et al.*, 1997; Pinkas-Kramarski *et al.*, 1998) is not represented. Note that MGF and VGF are narrow-specificity ligands, whereas SFGF and EGF have partially overlapping broad specificities. Nevertheless, wherever the two ligands overlap, SFGF-induced mitogenesis is superior to that of EGF. In addition, productive interaction of EGF with ErbB-4-expressing cells depends on overexpression of this receptor, or on co-expression of ErbB-2.

of the viral growth factors may be restricted to specific lineages of the host's cells. For example, the cells that undergo proliferation upon infection by the Shope fibroma virus (SFV) are undifferentiated fibroblasts, whereas the molluscum contagiosum virus affects primarily epidermal cells. On the other hand, mostly derivatives of a monocyte-macrophage lineage, called histiocytes, are found in the proliferative lesion of the Yaba virus (Sproul *et al.*, 1963). Because ErbB-1 is expressed by most fibroblasts and epithelial cells, but is absent in myeloid cells, whereas ErbB-3 and ErbB-4 are relatively restricted to epithelial cells, receptor specificity may be relevant to virus pathogenesis. Especially relevant is the shared specificity of SFGF and MGF to the ErbB-2/ErbB-3 heterodimeric receptor. This heterodimer is probably the most stable and potent receptor combination of the ErbB family (Pinkas-Kramarski *et al.*, 1996; Tzahar *et al.*, 1996), and its strong proliferative signals presumably underlie the oncogenic effect of an overexpressed ErbB-2 in epithelial tumors (Alimandi *et al.*, 1995; Wallasch *et al.*, 1995). Further, we have previously shown that skin keratinocytes are potently stimulated to proliferate when this heterodimer is induced by NDF (Marikovsky *et al.*, 1995). It is relevant, however, that replacement of the MGF-encoding sequence of the Myxoma virus with that of SFGF, TGFα or VGF resulted in myxomatosis that was clinically and histopathologically indistinguishable from that of the wild-type virus (Opgenorth *et al.*, 1993). Whether or not symptoms related to the fine specificity of each growth factor do not affect pathogenicity, or they simply escaped detection, remains an open question.

Enhancement of signaling by attenuation of receptor inactivation

How does a viral ligand (e.g. SFGF and MGF), whose receptor-binding affinity is significantly lower than that of the mammalian counterpart (e.g. EGF and NDF, respectively), transmit signals that are superior or comparable with that of the physiological ligand? Our results, which are relevant primarily to the SFGF–EGF pair, imply that rather than developing strategies to enhance signal generation, poxviruses evolved mechanisms that attenuate the rapid receptor inactivation process that follows ligand-induced receptor stimulation. In support of this model, ErbB-1 phosphorylation after SFGF binding is relatively low, but it is nevertheless sufficient for full activation of the MAPK (Figure 8A). Moreover, whereas MAPK stimulation by EGF is transient, sustained activation is achieved by SFGF, and the active kinase form is retained in the nucleus for a longer time (Figure 8B). In analogy with the relationships between viral and mammalian ligands, it has been shown repeatedly that transient activation of MAPK in murine fibroblasts results in cell growth, whereas sustained activation leads to cell transformation (reviewed in Marshall, 1995). It is interesting to note that receptor phosphorylation and MAPK activation deviate from precise quantitative coupling (Figure 8). This may reflect signal amplification downstream of the receptor, existence of spare receptors, or the fact that MAPK and ErbB-1 are subject to inactivation by different mechanisms. Besides dephosphorylation, which was not examined by us, receptor downregulation by means of endocytosis is a major process that attenuates growth factor signals (Sorkin and Waters, 1993). This process, however, is very inefficient in the case of the viral ligands (Figure 9). Recent observations made in several signaling systems, including that of ErbB-1 (Galcheva-Gargova *et al.*, 1995), imply that the ubiquitin-proteasome machinery plays a role in directing endocytosed receptors to intracellular degradation (reviewed in Hicke, 1997). This machinery is less efficiently recruited by a viral ligand, SFGF (Figure 8C), and the associated processes of receptor downregulation (Figure 9) and degradation (Figure 8C, lower panel) are severely attenuated. The mechanism underlying attenuation of receptor downregulation is unclear: according to one possibility, tyrosine phosphorylation of ErbB-1, which is conditional for internalization (Glenney *et al.*, 1988) and ubiquitination (Galcheva-Gargova *et al.*, 1995), is insufficiently high in the case of SFGF. Alternatively, although SFGF does not induce formation of large endocytic vesicles containing ErbB-1 (Figure 9C), it is still possible that this ligand, like another potent ErbB-1-specific ligand, namely TGFα (Ebner and Derynck, 1991), targets the receptor to rapid recycling through relatively small endocytic vesicles.

The role of SFGF in cellular hyperplasia near the sites of viral replication is best exemplified by deletion of the SFGF-encoding sequence from the genome of the malignant rabbit fibroma virus (MRV), which resulted in significantly less fatal syndrome and tumors with fewer proliferating cells (Opgenorth *et al.*, 1992). In fact, introduction of DNA of the Shope fibroma virus (SFV) into NIH 3T3 cells transformed these cells in culture, and they became capable of generating tumors in nude mice (Obom and Pogo, 1988). It is therefore conceivable that by

evading signal inactivation SFV potentiated its virulence. Presumably, the extremely low affinity of SFGF to ErbB-1 prevents the rapid endocytic clearance that normally follows growth factor binding to the cell surface, thereby extending the half-life of SFGF at the site of viral infection and replication. Although this mechanism has not been described before with any known EGF-like growth factor, a mutant of EGF (Y13G), engineered for enhanced mitogenic potency, displayed a 50-fold lower binding affinity, slow rate of depletion, and attenuated receptor downregulation (Reddy *et al.*, 1996). Also consistent with the proposed explanation of SFGF potency is the observation that a non-internalizing mutant of ErbB-1 can transform cells once stimulated by EGF (Wells *et al.*, 1990). Likewise, blocking ErbB-1 internalization by mutagenesis of dynamin, a guanosine triphosphatase that is required for clathrin vesicle formation, enhanced EGF-induced mitogenesis (Vieira *et al.*, 1996). We speculate that by adopting a strategy aimed at slow clearance of SFGF and sustained receptor activation, SFV eluded the need for a strong promoter and continuous transcription of the growth factor gene by host cells.

The mechanism underlying the relatively high mitogenic potencies of the other two viral ligands may differ from that of SFGF. The following reasons lead us to propose that VGF, in analogy to TGFα, induces recycling, rather than lysosomal degradation, of its receptor. First, ErbB-1 undergoes only partial downregulation after binding of VGF (Figure 9A). Secondly, receptor recycling is predictable because binding of VGF is relatively sensitive to the low pH characteristic of the maturing endosome, and downregulation of ErbB-1 by VGF is enhanced by monensin, a drug that blocks vesicular transport to the plasma membrane (our unpublished results). The potent action of MGF through the ErbB-2/ErbB-3 heterodimer appears to reflect yet a different mechanism, because the rates of internalization of ErbB-2 and ErbB-3 are much slower than the rate of endocytosis of ErbB-1 (Baulida *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996; Waterman *et al.*, 1998). Clarification of these and other emerging questions that relate to the pathogenesis of poxviruses will require further research. However, in the light of the possibility that all EGF-like ligands carry two receptor binding sites (Tzahar *et al.*, 1997), the data we presented may be useful for the design of ErbB-specific antagonists. In addition, our results underscore the potential physiological importance of low-affinity mammalian ligands, which commonly escape detection, in hyperproliferative and malignant states.

Materials and methods

Materials, buffers and antibodies

EGF (human recombinant) was purchased from Sigma. Epiregulin and a 47 amino acid-long fragment of EGF were synthesized as described (Shelly *et al.*, 1998). A recombinant form of NDF-β$1_{177–246}$ was from Amgen (Thousand Oaks, CA), and a longer form, HRGβ65 was synthesized as described (Barbacci *et al.*, 1995). Polyclonal rabbit anti-c-Cbl (C-15) antiserum and a monoclonal anti-phosphotyrosine antibody (PY-20) were purchased from Santa-Cruz Biotechnology (Santa-Cruz, CA). Antibodies to ubiquitin were kindly provided by Dr Sadaki Yokota. Anti-Shc antibodies were from USB (Cleveland, OH). mAb111 and mAb199 directed to the extracellular domain of ErbB-1 were generated in our laboratory essentially as described (Chen *et al.*, 1996). A polyclonal anti-ErbB-1 antibody (anti-C-terminal) was raised against a 14 amino acid-long peptide that corresponded to the C-terminal sequence of the ErbB-1. For detection of activated MAP-kinase we used a mAb directed to the doubly phosphorylated form of MAP-kinase in which both tyrosine and threonine residues of the TEY motif were phosphorylated (Yung *et al.*, 1997). Binding buffer contained RPMI medium with 0.2% bovine serum albumin (BSA). HNTG buffer contained 20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol. Solubilization buffer contained 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1.5 mM EGTA, 2 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin and leupeptin, each at 10 μg/ml.

Synthesis of viral peptides

Peptides corresponding to the EGF-like motif of the three viral proteins were synthesized on an Applied Biosystems (ABI) model 431 peptide synthesizer fortified with UV feedback monitoring at 301 nm, and using FMOC-Rink amide AM resin. The conventional ABI monitor-previous-peak-algorithm was employed up to five times with a cut-off of 3.5% of the first deprotection. A secondary deprotection [using 2%DPU/2% piperidine/96% *N*-methylpyrolidone (NMP)] was performed and followed by double coupling. Acetic anhydride/hydroxybenzotriazole (HOBT) capping was utilized at the end of each coupling, followed by washing with 1:1 trifluoroethanol/dichloromethane (DCM). The peptides were deprotected and removed from the resin as described (King *et al.*, 1990), with the following modifications: methoxyindole (2%) was added to reagent K, and the reaction time was changed to 3.5 h. Small quantities of the reduced peptides were purified by reverse-phase high performance liquid chromatography and examined by matrix-assisted laser desorption ionization (MALDI) mass spectral analysis. The crude reduced proteins were dissolved in a Tris–HCl buffer pH 6.0 containing 6 M guanidium–HCl and diluted to a concentration of 0.06 mg/ml in methionine-containing buffer (10 mM) that included 1.5 mM cystine, 0.75 mM cysteine, and 100 mM Tris pH 8.0. The mixture was stirred for 48 h cold, and the oxidized peptide isolated on a C-4 VYDAC 10 micron preparative column (22×250 mm) using a 0.1% trifluoroacetic acid-water/acetonitrile gradient. $EGF_{1–47}$ was similarly synthesized, starting at the seventh residue of the 53 amino acid-long EGF sequence. The oxidized proteins were lyophilized and characterized by mass spectrometry and amino acid analysis, and shown to be homogeneous. Electro-spray mass spectrometry was used to verify the mass of the synthetic peptides. Disulfide bonding patterns were analyzed by using a mixture of proteolytic enzymes (trypsin and Glu-C) and MALDI mass spectrometry. The canonical pattern of the EGF-like motif (Cys1-Cys3, Cys2-Cys4 and Cys5-Cys6) was confirmed.

Cell lines and tissue culture

Derivatives of the 32D murine hematopoietic progenitor cell line were grown in RPMI-1640 medium supplemented with antibiotics, 10% heat-inactivated fetal bovine serum (FBS), and 0.1% medium that was supplied with IL-3. The various sublines expressing ErbB combinations were described previously (Pinkas-Kramarski *et al.*, 1996), except for the ErbB-4-expressing line (D4), whose level of expression was ~10-fold higher than in D24 and in D34 cells, due to the use of different promoters (denoted E4 cell line in Alimandi *et al.*, 1997). Chinese hamster ovary (CHO) cells expressing combinations of ErbB proteins were previously described (Tzahar *et al.*, 1996). These cells were grown in DMEM/F12 (1:1) medium supplemented with antibiotics and 10% heat-inactivated FBS.

Cell proliferation assays

Cells were washed free of IL-3, resuspended in RPMI-1640 medium supplemented with calf serum (10%) at 5×10^5 cells/ml, and treated without or with growth factors or IL-3 (1:1000 dilution of medium conditioned by IL-3-producing cells). Cell survival was determined by using the [3-(4,5-dimethylthiazol-z-yl)-2.5 diphenyl] tetrazolium bromide (MTT) assay as previously described (Pinkas-Kramarski *et al.*, 1996). MTT (0.1 mg/ml) was incubated for 2 h at 37°C with the analyzed cells. Living cells can transform the tetrazolium ring into dark blue formazan crystals, that can be quantified by optical density at 540–630 nm after lysis of the cells with acidic isopropanol (Mosman, 1983). For dose–response analyses, serial dilutions of the ligands were added in RPMI-1640 medium, and cells were harvested 24 h after plating. For cell survival experiments, cells were treated without or with various ligands (100 ng/ml) or IL-3, and survival determined 24, 48 or 72 h later.

Ligand binding and covalent crosslinking analyses

Growth factors were labeled using Iodogen as described (Karunagaran *et al.*, 1995). The specific activity ranged between 0.5 and 5×10^5 c.p.m./ng.

Ligand displacement analyses with 32D cells were performed as described (Pinkas-Kramarski *et al.*, 1996) with the following modifications: cells were washed once with binding buffer, divided into tubes (final volume 0.1 ml) and then incubated for 2 h at 4°C with the radiolabeled EGF, or NDF, and various concentrations of unlabeled ligands. Nonspecific binding was determined in the presence of the corresponding unlabeled ligand at 1 μg/ml. Ligand binding to CHO cells was performed as described (Tzahar *et al.*, 1996). Chemical crosslinking experiments of the viral peptides were performed as follows: the various 32D cell lines (1×10^7 cells) were incubated for 2 h on ice with the radiolabeled ligand (250 ng/ml). The chemical crosslinking reagent bis(sulfosuccinimidyl) substrate (BS^3) was then added to 1 mM final concentration. After 45 min at 4°C cells were washed with phosphate-buffered saline (PBS). Cell lysates were prepared and analyzed by immunoprecipitation using mAbs against the four ErbB proteins, or control mAbs, as described (Tzahar *et al.*, 1996). This assay was also performed in the presence of mAbs against ErbB-1, either mAb111 or mAb199, each at 20 μg/ml. In this case, the immunoprecipitation step was performed using a polyclonal rabbit antibody to ErbB-1.

Lysate preparation and immunoprecipitation

Cells were exposed to the indicated stimuli in RPMI-1640 medium or in binding buffer. After treatment, cells were collected by centrifugation and solubilized in lysis buffer. Lysates were cleared by centrifugation. For direct electrophoretic analysis, boiling gel sample buffer was added directly to the cells and the lysates were mixed vigorously to brake genomic DNA. For crosslinking experiments, lysates were first subjected to immunoprecipitation with various antibodies. Rabbit antibodies were directly coupled to protein A–Sepharose while shaking for 1 h at 4°C. Mouse antibodies were first coupled to rabbit-anti-mouse immunoglobulin G and then to protein A–Sepharose by using the same procedure. The proteins in the lysate supernatants were immunoprecipitated with aliquots of the protein A–Sepharose–antibody complex for 2 h at 4°C. The immunoprecipitates were washed three times with HNTG, and resolved by SDS–polyacrylamide gel electrophoresis (PAGE). Either 6% acrylamide gels (crosslinking assays) or 8.5% acrylamide gels (receptor phosphorylation and MAPK assays) were used. Proteins were then electrophoretically transferred to nitrocellulose membranes that were blocked for 2 h in TBST buffer (0.02 Tris–HCl pH 7.5, 0.15 M NaCl and 0.05% Tween 20) containing 5% milk, and blotted with primary antibodies for 2 h. This was followed by a secondary antibody linked to horseradish peroxidase. For receptor phosphorylation and MAPK activation experiments, the upper part of the membrane was used for immunoblotting with an anti-phosphotyrosine mAb, and the lower part of the membrane was used for immunoblotting with a mAb to the active form of MAPK. Immunoreactive bands were detected with an enhanced chemiluminescence reagent (Amersham Corp).

Receptor downregulation assays

CB1 cells grown in 24 well plates were washed with binding buffer and then incubated at 37°C for up to 3 h with various ligands to allow receptor downregulation. Cells were then put on ice, rinsed twice with binding buffer and surface-bound ligand removed by acid wash (0.5 ml solution of 150 mM acetic acid at pH 2.7, containing 150 mM NaCl). The receptor level was then determined by binding of radiolabeled EGF (5 ng/ml) to the cells for 2 h at 4°C. Cell-associated radioactivity was determined after collecting a second acid wash.

Immunofluorescence

CB1 cells were plated on glass coverslips and grown for 24 h. Cells were rinsed with serum-free medium and then treated with a growth factor (250 ng/ml) for 5 min at 37°C. Treated coverslips were fixed with 3% paraformaldehyde in PBS (15 min), and then rinsed with PBS. Cells were permeabilized for 10 min at 22°C with PBS containing 1% BSA and 0.2% Triton X-100. This was followed by a 10 min incubation with PBS. For immunodetection of ErbB-1, coverslips were incubated for 1 h at 22°C with mAb111. After extensive washing with PBS, the coverslips were incubated with a Cy3-conjugated goat-anti-mouse [$F(ab')_2$ specific] antibody (Jackson ImmunoResearch Laboratories) and washed three times with PBS. Coverslips were viewed with a Zeiss fluorescence microscope in oil immersion. For immunodetection of activated MAPK we used D1 cells. After stimulation with ligands (at 50 ng/ml) cells were washed with PBS, spun onto a glass slide, and fixed in 3% formaldehyde in PBS solution for 30 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, and reacted with a mAb directed to the active form of MAPK (Yung *et al.*, 1997). Following extensive washing with PBS, antibody detection was performed using rhodamine-conjugated goat anti-mouse IgG (1:1000, Jackson ImmunoResearch Laboratories). To visualize nuclear DNA, cells were stained with 4,6-diamidino-2-phenylindole (DAPI, dissolved in PBS).

Acknowledgements

We thank Sara Lavi for technical assistance, Rony Seger for help with the MAPK assays, and Avraham Amsterdam for fluorescent microscopy. This work was carried out with financial support from the Department of the Army (grant DAMD17-97-1-7290) and from The Israel Science Foundation. S.S. is the recipient of a fellowship of the Telethon Muscular Dystrophy Fund (Italy).

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Received February 26, 1998; revised August 17, 1998; accepted August 25, 1998