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Research Article

Inhibition by transmembrane peptides of chimeric insulin receptors

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Abstract. Receptor tyrosine kinases play essential roles in cell proliferation and differentiation. We have recently shown that peptides corresponding to the transmembrane domains of the epidermal growth factor (EGF) and ErbB2 receptors inhibit their corresponding receptor activation in cancer cell lines. We extend this observation to cells transfected with chimeric insulin receptors where the transmembrane domain has been replaced by that of the EGF receptor or a mutated Erb2 domain. Peptides corresponding to the transmembrane domains of the EGF receptor and ErbB2 are able to inhibit specifically the autophosphorylation of insulin receptors with the corresponding domain. This inhibitory effect is correlated with the propensity of the different transmembrane domains to self-associate in a genetic reporter assay. Thus, our data strengthen the notion that transmembrane domains are involved in erbB receptor activation, and that these receptors can be modulated by inhibiting protein-protein interactions within the membrane.

Key words. Receptor tyrosine kinase; dimerization; hydrophobic peptide; insulin receptor; EGF receptor; ErbB2.

Receptor tyrosine kinases (RTKs) are transmembrane (TM) glycoproteins that consist of a variable extracellular N-terminal domain, a single membrane-spanning domain, and a large cytoplasmic portion composed of a juxtamembrane domain, the highly conserved tyrosine kinase domain, and a C-terminal regulatory region [1]. Dimerization is necessary for RTK activation [2]. Nevertheless, recent data have shown that receptor dimerization may not be sufficient for activation. This notion stems mostly from experimental modifications of the TM domain of some RTKs and other related receptors [3]. Although this domain was initially thought to be a merely passive anchor of the receptor into the membrane bilayer, a Val → Glu point mutation in the TM domain of one RTK, (neu/ErbB2) was found to enhance its transforming properties through increased dimerization and constitutive activation of signaling [4]. This increased dimerization is thought to be mediated by direct interactions between the TM domains. Indeed, the TM domains of all four receptors of the ErbB/HER RTK family have been shown to have a strong propensity to interact and dimerize [5].
Furthermore, we have recently reported the effect of the expression of hydrophobic TM peptides on the activation of ErbB2 and epidermal growth factor (EGF) receptors overexpressed in human cancer cells [6]. Our

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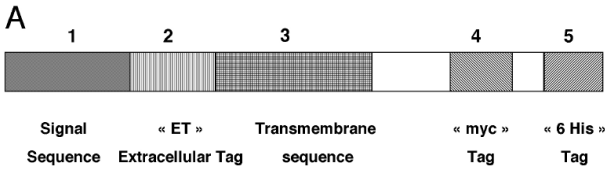
results demonstrated that expression of peptides corresponding to the TM domain of these receptors is able to inhibit specifically the corresponding receptor activation in whole cells. To demonstrate further that this inhibition is exclusively due to TM-TM domain interactions, we decided to test the effect of TM peptides on chimeric insulin receptors (IRs) where the TM domain had been replaced by that of the EGF receptor (EGFR) or by the Val → Glu mutated neu/ErbB2 TM domain (ErbB2m). Our results show that expression of peptides corresponding to the TM domain of EGFR and ErbB2m can inhibit specifically chimeric IRs with the corresponding TM domain while expression of peptides corresponding to the IR TM domain does not inhibit activation of wild-type or chimeric IRs in whole cells. Furthermore, this inhibition of activation of chimeric IRs by expression of peptides is accompanied by an inhibition of AKT, an enzyme which belongs to signaling pathways coupled to the IR and which is implicated in cell proliferation. We also attempted to correlate these effects of TM peptides with their propensity to autodimerize in the Toxcat bacterial assay [7] and found that the ability of TM peptides to inhibit kinase activity of wild-type or chimeric IRs is proportional to their ability to form dimers.



B

InsR	IIIGPLIFVFLFSVVIGSIYLFLRKR
EgfR	SIATGMVGALLLLLVVALGIGLFMRRR
erbB2^{mut}	FIIATVEGVLLFLILVVVVGILIKRR
erbB2	SIISAVVGILLVVVLGVVFGILIKRR

Figure 1. Schematic diagram of the minigenes used to encode the transmembrane sequences (A). Each box represents a section of the constructions made within the Invitrogen pSecTag A plasmid. Amino acid sequences were as follows (one-letter code): signal sequence peptide (METDTLLLWVLLLWVPGSTG, 1–20), artificial extracellular sequence (extracellular tag, ET) from the multiple cloning site (DAAQPARRAVRSF, 21–33), different transmembrane regions as indicated (B) including the stop transfer tribasic sequence (as indicated, 34–60), followed by the intracellular domain (HPAQWRPLESRGPEQKLISEEDLNSAVDHHHHHH, 61–94) containing the myc and polyHis tags. Putative dimerization domains of the type GXXXG (two glycines separated by any three residues), or smallXXXsmall (two small residues separated by any three residues), are underlined in each sequence (see Discussion), and the tribasic stop sequence is italicized. Position of the mutant glutamic acid is shown in bold in the ErbB2^{mut} sequence [4].

Materials and methods

Plasmid construction. The expression plasmid encoding the chimeric IR was prepared as previously described [8] from the PetNdeI plasmid. It contains the entire cDNA sequence of the human IR with the exception of the TM domain sequence (corresponding to amino acids Lys^{928} to Gln^{956}), which was deleted by site-directed mutagenesis and replaced by a unique *Nde*I restriction site. The chimeric receptors (Pet IR-EGFR and Pet IR-ErbB2m) were constructed with complementary oligonucleotides phosphorylated and annealed to form a linker representing the wild-type TM sequence of the human EGFR or the transforming Val → Glu-mutated sequence of the rat neu/ErbB2 receptor [4].

Expression vectors for the short TM domains were constructed with pSecTagA vectors (Invitrogen) as described elsewhere [6]. This vector contains a leader sequence, a multiple cloning site, and two tag sequences [myc and polyhistidine (polyHis)]. The minigenes encoding the short TM sequence peptides (of the IR, EGFR, mutated rat ErbB2, or wild-type human ErbB2) were constructed by ligation of synthetic oligonucleotides, using the *Eco*RV and *Hind*III restriction sites. The final sequence comprised the signal peptide and an adjacent extracellular sequence joined to a sequence incorporating the entire putative TM domain and C-terminal intracellular tag sequences (Myc and polyHis) (fig. 1). All plasmids were verified by sequencing.

Transfection of cDNAs and selection of cell lines. Chinese hamster ovary (CHO) cells were cultured in Ham's F12 medium containing 10% fetal calf serum as described previously [8], transfected with Pet IR-EGFR or Pet IR-ErbB2m plasmids, and selected by fluorescence-activated cell sorting as previously described [8]. The resulting cell lines which overexpress chimeric IRs with an EGFR or ErbB2m TM were termed IR-CHO-EGFR^{TM} and IR-CHO-ErbB2m^{TM}, respectively. IR-CHO-wt cells (transfected with the native human IR cDNA) were prepared and selected in parallel.

For peptide expression studies, all three CHO cell lines were transiently transfected at 50–60% confluence by lipofectamine (Invitrogen) using 2 µg pSecTag plasmid DNA encoding the EGFR, ErbB2m, ErbB2 or IR TM peptide.

^{125}Insulin-binding assays, and purification of receptors. Insulin binding on whole cells, solubilization and wheat germ agglutinin (WGA) purification of receptors were performed as previously described [8].

Antibodies for extracellular tag detection. The extracellular tag (ET) antibody was produced after immunization of rabbits with a synthetic amino acid sequence (DAAQPARRAVRSFC) conjugated to keyhole limpet

hemocyanin (Eurogentec). This sequence corresponds to the remaining 5' part of the pSecTag plasmid multiple cloning site, and has no homology with any sequence found in databases. The polyclonal antibodies were purified (CAP-8 IgG Purification Kit; Sterogene) before use.

Western blotting. After incubation with 10^{-8} M insulin for 5 min, cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested in lysis buffer [20 mM Tris pH 8, 137 mM sodium chloride, 1% (v/v) Nonidet P-40, 10% (v/v) glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate] supplemented with a protease inhibitor cocktail (Roche) and 5 mM sodium orthovanadate. Cell lysates were cleared by centrifugation at 14,000 g for 10 min at 4 °C. Proteins in total lysates or WGA-purified receptors were assayed before SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using the BCA Protein Assay Kit (Pierce) with bovine serum albumin as a standard. After SDS-PAGE separation, proteins were transferred onto nitrocellulose membrane (Whatman), blots were blocked overnight, and then incubated for 2 h with primary antibody: anti-IR antibody (1 µg/ml; Neomarkers, clone CT-3), anti-PTYR antibody (0.2 µg/ml; Upstate Biotechnology clone 4G10), anti-phosphorylated Akt antibody (1:2000 dilution; Cell Signaling Technology, ref. 9271S), or anti-myc antibody (1 µg/ml; NeoMarkers, clone 67P05). The membranes were then washed and incubated with peroxidase-labeled recombinant A/G protein (Pierce) diluted to 1:100,000 for 1 h. In some experiments, membranes were stripped of antibody (Restore Western blot stripping buffer; Pierce) and reprobed with a different antibody. Bands were visualized using the SuperSignal West Dura Extended Duration Substrate kit (Pierce), according to the manufacturers instructions, and recorded with a computerized Syngene GeneGnome imager.

Double labeling immunocytochemistry and confocal laser scanning microscopy. IR-CHO cells were transiently transfected as described above. Cells were grown on polylysine coated glass coverslips for 3 days. Cells were then rinsed with PBS, and subsequently fixed with 4% (w/v) paraformaldehyde. Following 6 rinses at 37°C, cells were incubated with 3% (w/v) serum albumin in PBS at 37 °C for 45 min to reduce non-specific staining. Coverslips were then incubated at room temperature for 2 h with primary antibody: ET antibody (20 µg/ml) or IR antibody (2 µg/ml; Neomarkers, clone 83-7). After six washes, cells were incubated for 60 min at 25 °C with cyanin 3 (CY3)-labeled anti-mouse antibody (1:2000 dilution; Jackson Immuno Research) or Alexa Fluor 488-conjugated anti-rabbit (1:300 dilution; Molecular Probes). After extensive washing, the coverslips were mounted in mowiol 4-88. Immunofluorescence staining was monitored with a Zeiss laser scanning microscope (LSM 510) equipped with a Pan-Achromat x63 oil immersion lens (numerical aperture 1.4). Alexa Fluor 488 emission was excited using the 488-nm ray of the argon laser, while CY3 was excited using the 543 nm line of the helium/neon laser. Emission signals of Alexa Fluor 488 and CY3 were filtered with a BP 505-530 and an LP 560 filter, respectively.

Synthesis and purification of hydrophobic peptides. Peptides corresponding to the TM domains of the EGF and insulin receptors (sequences SIATGMVGALLLLLVVALGIGLFMR and KIIIGPLIFVFLFSVVIGSIYLFLR, respectively) were synthesized and purified by Neosystems using FMOC chemistry. HPLC analysis (reverse phase C4 column) demonstrated that the peptides were ~98% pure, and they were checked by sequencing.

TOXLUC (TOXCAT) assays. To explore the ability of the studied isolated TM domain sequences to undergo homo-oligomerization, we employed a modified TOXCAT assay [7], which measures the association between TM helices in the *E.scherichia coli* inner membrane. The assay exploits a dimerization-dependent activator of transcription, called ToxR. The TM sequence of interest is expressed in the bacteria as a chimeric protein flanked by ToxR and by the maltose-binding protein (MBP). TM domain-mediated oligomerization results in ToxR-activated expression of a reporter gene encoding chloramphenicol acetyltransferase (CAT) in the original version of the system. For convenience, we used conventional molecular biology methods to replace the initial CAT gene by that of luciferase. Synthetic TM sequences corresponding to the IR, EGFR and mutant or wild-type ErbB2 were cloned into the new plasmid as NheI/DpnII fragments. Chimeras with TM sequences derived from glycophorin A or its G83I mutant [7] were used as controls. The luciferase assay was performed using the Roche assay kit, according to the manufacturers instructions, and a Berthold Microlumat plate luminometer.

Results

Characterization of the chimeric receptors. We first checked whether the replacement of the native TM domain of the IR with that of the EGFR and mutant ErbB2 had any effect on ligand binding and autophosphorylation. Analysis of ^{125}I-insulin binding in competition experiments showed that displacement curves were superimposable for all three types of cells studied (not shown). Total binding levels were equivalent, as expected, after fluorescence-activated cell sorting with anti-receptor antibodies. Kinase activity of the receptors was assayed in whole cells by anti-phophotyrosine Western blotting (fig. 2). Both wild-type and EGFR^{TM} chimeric receptors

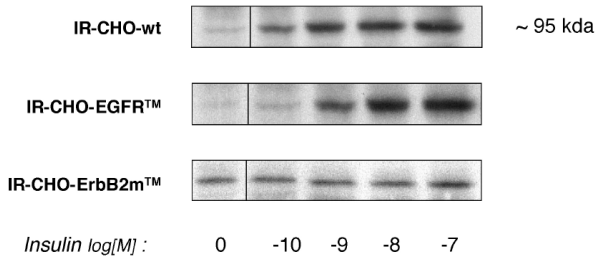


Figure 2. Insulin effect on the phosphorylation of its receptor in CHO cells transfected with the wild-type (IR-CHO-wt) or the chimeric IRs (IR-CHO-EGFR™ and IR-CHO-ErbB2m™). Cells were serum starved overnight and treated with the indicated concentration of insulin for 5 min. Cells were then lysed and equal amounts of protein were submitted to SDS-PAGE, followed by blotting with anti-phosphotyrosine antibody. A typical experiment is shown. The apparent molecular mass corresponding to the receptor beta subunit is shown on the right.

displayed a typical dose response upon insulin stimulation. In contrast, receptors from IR-ErbB2m™ cells displayed a markedly elevated basal activity in the absence of insulin, and a modest stimulation by the hormone, as previously shown by Cheatham et al. [9]. These results demonstrate that replacement of the TM domain of the IR by that of the EGFR has no major effect either insulin binding or signaling of this receptor. Previous mutagenesis studies of this TM domain have shown it to be quite tolerant to modifications [10, 11], except when the activating domain of mutant ErbB2 [9] or the inhibitory domain of glycophorin A [8] were introduced in place of the wild-type TM domain.

Expression of hydrophobic TM sequences. To test the potentially inhibitory effects of TM peptides on the activation of wild-type or chimeric IRs, we took advantage of the characteristics of the pSecTag expression plasmid. In this plasmid, we constructed mini-genes comprising a sequence signal at the N terminus, which should direct the protein to the cell surface, a short antigenic extracellular portion termed ET, the different TM sequences of interest, a basic short stop sequence to anchor the peptide in the proper orientation, and two C-terminal intracellular tags (myc and polyHis) (see fig. 1). Anti-myc tag antibodies were used in Western blotting experiments to control the synthesis of a peptide with correct apparent molecular mass (~9 kDa) after transient transfection in CHO cell lines. The 9 kDa fusion peptide expression was maximal at day 2 after transfection and was not detectable in non-transfected control cells (data not shown).
Immunolocalization of the fusion peptides via confocal laser microscopy and the anti- ET antibodies demonstrated their localization on the cell membrane. Figure 3 shows the labeling patterns in non-permeabilized (upper panels) and permeabilized (lower panels) cells with anti-ET antibodies and with both anti-ET and anti-receptors antibodies. A distinct bright outline labeling for all antibodies indicated the peripheral membrane localization of antigens. This was confirmed by the analysis of pictures taken at different scanning depths (Z-scans, not shown). IRs appeared to be quite evenly distributed around cells while the anti-ET labeling had an uneven, patched aspect (fig. 3B, D), possibly related to the tendency of the TM peptides to self-associate or aggregate. IR, EGFR and ErbB2m TM peptides yielded similar labeling patterns.

Effects of TM peptides on receptor expression, phosphorylation and signaling. The above-described experiments established that the expression plasmids encoded peptides with the correct apparent molecular mass and the expected membrane localization and orientation. We next assayed whether the expression of these fusion peptides had any effect on different functional aspects of the IR overexpressed in the CHO cell lines used here. To this end, we looked for a possible correlation between the expression of receptors and peptides, together with autophosphorylation of receptors and phosphorylation of one of the major kinases in signaling pathways, namely Akt. This was done by Western blot analysis of cell extracts at day 2 after transfection, using antibodies directed against the insulin receptor, the myc tag, and antibodies specific to phosphotyrosine and to the phosphorylated form of Akt. Equivalent amounts of protein were used to allow comparison between samples, and this was verified by measuring the amount of actin in each sample.
Typical results are shown in figure 4A. For the three CHO cell lines and all peptide-encoding plasmids, IR expression and actin level were constant. Anti-myc antibodies revealed the absence of TM peptide in non transfected cells and its presence in transfected cells, however at variable levels. Anti-phosphotyrosine antibodies analysis of IR autophosphorylation showed no detectable peptide effect for the wild-type IR-CHO-wt cells. Conversely, expression of the EGFR TM peptide induced a decrease in receptor phosphorylation in IR-CHO-EGFR™ cells, and mutated rat ErbB2 TM peptide (ErbB2m) also decreased the receptor phosphorylation in IR-CHO-ErbB2m™ cells. In this latter cell line, a human ErbB2 peptide was also effective, but less than the mutant sequence. Very similar peptide-induced effects were observed for the phosphorylation of the intracellular kinase Akt.
Figure 4B summarizes the data from three to four independent experiments. Black and hatched histogram bars represent IR and Akt phosphorylation, respectively. The wild-type IR peptide clearly had no effect while only TM peptides corresponding to the TM domain of the chimeric receptor constructs inhibited phosphorylation. The EGFR TM peptide produced the maximal effect on IR-CHO-EGFR™ cells with about 60% inhibition. The mutant ErbB2 TM peptide induced an approximately

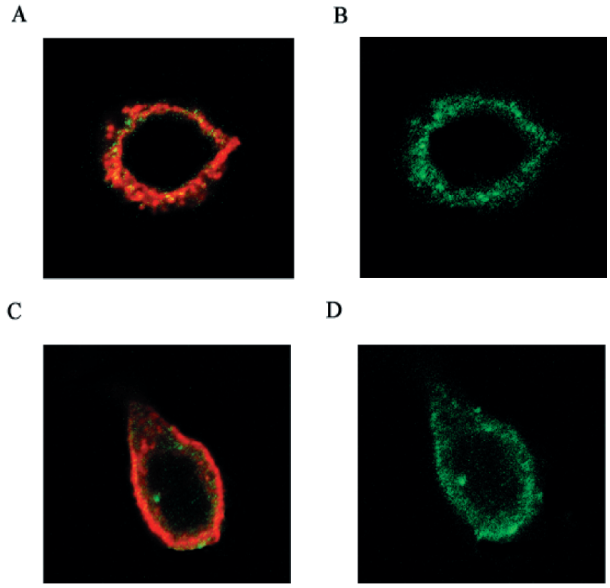


Figure 3. Immunofluorescence analysis of whole cells expressing the transmembrane construct using anti-receptor and anti-tag antibodies. Staining of intact (A, B) or permeabilized (C, D) IR-CHO cells with anti-ET (green, B, D) or anti-IR (red, A, C) together with anti-ET (green) antibodies (showing intense membrane staining, revealing the expression of transmembrane peptides at the cell surface, together with the receptors.

50% inhibition of receptor phosphorylation in IR-CHO-ErbB2m^{TM} cells, whereas the wild-type human ErbB2 TM peptide was only ~15% inhibitory.

Synthetic peptides effects. Our results demonstrate that membrane expression of EGFR and ErbB2m TM fusion peptides provoke a specific inhibition of the autophosphorylation and subsequent signaling of chimeric IRs containing the same TM domain. Although the expression of the transfected peptides varied slightly between experiments, the observed inhibitory effects were proportional to the amount of detectable peptide. Notably, time-course experiments showed inhibition to be maximal at day 2 after transfection, the day at which maximal expression of the peptide was observed (data not shown). However, our experimental design with transient transfection did not allow measurement of the amount of effective peptide. In an attempt to gain more quantitative information, similar experiments were carried out using synthetic peptides. Shorter peptides corresponding to the hydrophobic core of the TM domains of insulin and EGF receptors, without tag sequences, were chemically synthesized and purified by reverse-phase HPLC. Peptides were then incorporated in detergent micelles and added to the WGA purified solubilized receptors prepared from both IR-CHO-wt and IR-CHO-EGFR^{TM} cells.

The effect of incorporated peptide on receptor phosphorylation was measured after 1 h incubation with peptides, stimulation with 10^{-8} M insulin for 5 min in the presence of 30 μM ATP, followed by SDS-PAGE and Western blotting with anti phosphotyrosine antibodies. Figure 5 presents the results of scanning quantitation of two experiments. Again, the IR peptide (black bars) had no effect at any concentration tested, and no effect was seen in IR-CHO-wt cells (fig. 5A) with either IR or EGFR (hatched bars) TM peptides. In IR-CHO-EGFR^{TM} cells (fig. 5B), no or very modest effects were observed at the higher concentration (10^{-7} M) of EGFR peptide tested. In contrast, a striking decrease by ~50% and a mild decrease by ~15% of receptor autophosphorylation was observed at 10^{-9} M and 10^{-8} M of EGFR TM peptide. The absence of effect at the higher peptide concentration is most certainly due to the observed tendency of the peptides to aggregate or self-associate in the membrane, and is in close agreement with our previous results in A431 cells [6].

Study of TM domains dimerization in E. coli membranes. Altogether, these results clearly demonstrate that the inhibition of phosphorylation is due to interactions at the TM level, and that this effect is independent of the extracellular and cytoplasmic regions of the receptor. To further explore the self-association potential of the TM sequences, we employed TOXLUC, a modified TOXCAT system [7], in which a luciferase system replaces the CAT reporter gene (see Materials and Methods). Transformation was performed in DH5alpha or MM39 E. coli cells. Membrane insertion of chimeras was verified by survival on M9-maltose medium for MM39 cells (not shown), indicating periplasmic localization of the MBP domain.

The results using the TOXLUC assay for sequences derived from the IR, EGFR, wild-type ErbB2, and Val → Glu mutant ErbB2 TM domains are depicted in figure 6. These studies demonstrate that all three sequences have some tendency to form homo-oligomers, but to a lesser extent than the glycophorin A TM sequence, known to possess a strong tendency for homodimerization. For comparison, the signal obtained with a glycophorin A TM sequence containing a mutation that destabilizes the dimer (G83I) is shown. Notably, the TM domain of the IR presents a low, but significant, dimerization capability. ErbB2 and ErbB2m come next, and the EGFR TM domain has the best ability to form a dimer. Our results with TM peptides of the EGFR family are in good agreement with previously reported data [5, 12]. Of particular interest, the peptide dimerization data correlated well with the peptide effects on the chimeric receptor kinase activity and signaling. Expression levels of the chimeras were assayed using anti-MBP immunoblotting, and whole-cell lysates were found to contain identical amounts of ToxR-TM-MBP fusion proteins (data not shown).

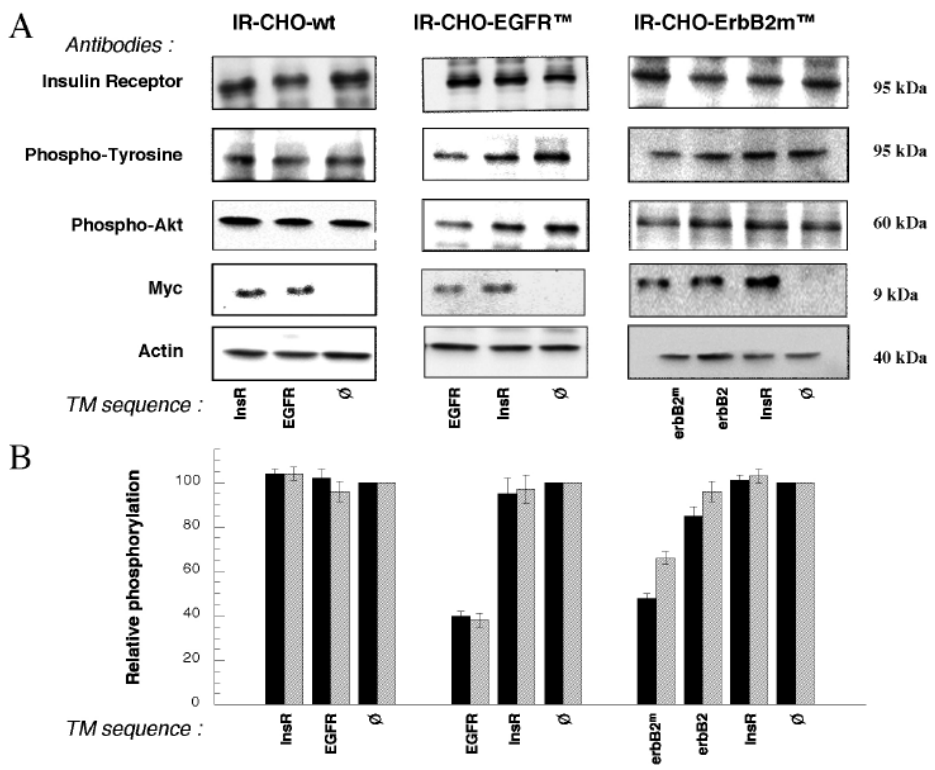


Figure 4. Expression, autophosphorylation and signaling of IRs in transfected CHO cells. Experiments were performed at day 2 after transfection with pSecTag plasmids encoding the TM peptides as indicated, for IR-CHO-wt, IR-CHO-EGFR™ and IR-CHO-ErbB2m™ cells (from left to right as shown at the top). Cells were serum starved overnight and treated with 10^{-8} M insulin for 5 min before lysis. Identical protein amounts of cell lysates were subjected to SDS-PAGE and Western blotting using antibodies as indicated on the left. (A) Typical experiment out of three to four similar ones. (B) Combined results after computer analysis of blots, as histograms (mean ± SE) for IR (black bars) and Akt (hatched bars) phosphorylation. Values in non-transfected cells are taken as 100%.

Discussion

In the present paper, we examined the consequence of introducing peptides corresponding to the TM sequences of the insulin, EGF, and ErbB2 receptors on the signaling activity of TM domain-substituted chimeric IRs. This work is an extension of a recently published paper [6] in which we have shown that expression vectors encoding short fusion peptides encompassing native or mutated TM domains of the EGF and ErbB2 receptors were able to inhibit specifically the autophosphorylation and signaling pathway of their cognate receptor in human cell lines overexpressing the wild-type EGFR or ErbB2.

The IR TM domain has been shown to be quite tolerant to TM domain alterations [10]. Thus, it is not surprising that replacement of the native TM sequence by that of the EGFR did not alter insulin binding, receptor autophosphorylation, or Akt activation by insulin. Such tolerance for that precise TM domain could also be inferred from other chimeric receptors consisting of the IR extracellular domain with the TM and kinase domains of the EGFR, or the TM and extracellular domains of EGFR with the insulin receptor kinase domain [13]. These hybrid proteins were found to be fully functional, showing that the two major extra- and intracellular domains of this class of receptor are able to interact across the membrane, thereby indicating a common scheme for TM signaling. Nevertheless, the substitution of the IR TM domain by that of the EGFR may quite possibly induce more subtle changes in insulin signaling than the few parameters studied here.

In contrast, substitution of the IR TM domain by that of the mutated (Val → Glu) oncogenic ErbB2 [4] resulted in a constitutively active IR kinase, which was hardly, if at all, sensitive to insulin. This is in excellent agreement with a previous report on a similar construction [9]. In addition, it should be pointed out that this peculiar TM domain or the introduction of similar mutations in other TM domains have been found to cause constitutive activation in other receptor tyrosine kinases [14–17], again suggesting a common mechanism for receptor activation.

Peptides corresponding to the TM domains of EGFR and ErbB2 were found to specifically inhibit the autophosphorylation of chimeric IRs with the corresponding domains, while the TM domain of the IR was unable to do so. We also found that this inhibitory effect is roughly correlated with the propensity of the different TM domains to self-associate in a bacterial genetic reporter assay. These results are in good agreement with those previously obtained on native EGFR and ErbB2 receptors in cancer cells [6].

Nevertheless, some of our results are apparently paradoxical, especially since the IR is already a covalent dimer. First, the absence of effect of the IR TM peptide is inconsistent with previous results demonstrating the importance of this domain in receptor activation. Notably, replacement of this domain by the known dimerizing TM

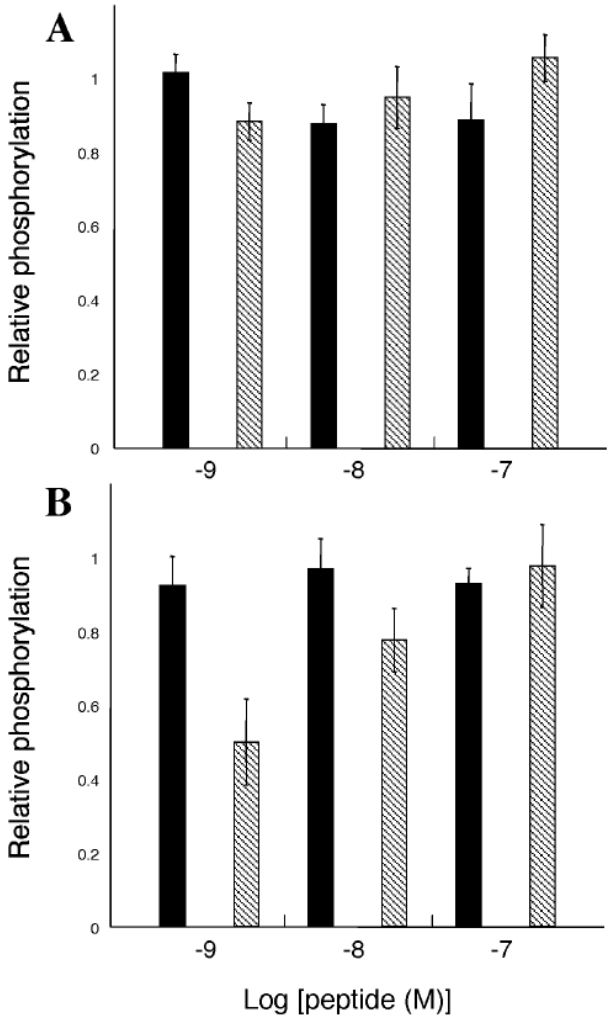


Figure 5. Autophosphorylation of IRs solubilized from IR-CHO-wt and IR-CHO-EGFR^{TM} cells and incubated with synthetic TM peptides. Peptides corresponding to the hydrophobic core of the TM domain of insulin and EGF receptors were chemically synthesized and purified, incorporated in detergent micelles and incubated with WGA-purified receptors for 1 h. After 5 min incubation in the presence of 10^{-8} M insulin and 30 μM ATP, equal amounts of protein were submitted to SDS-PAGE and Western blotting with anti-IR and anti-phosphotyrosine antibodies. Densitometric analysis of blots was performed and results normalized according to the amount of immunoreactive IRs in each sample, and expressed as percentage of the observed autophosphorylation in the absence of peptides (taken as 100%). Results are depicted as histograms for the effects of IR (black bars) and EGFR (hatched bars) TM domains at three concentrations on receptors prepared from IR-CHO-wt (A) and IR-CHO-EGFR^{TM} (B) cells. The figure represents the results (mean ± SE) of two similar experiments performed in duplicate.

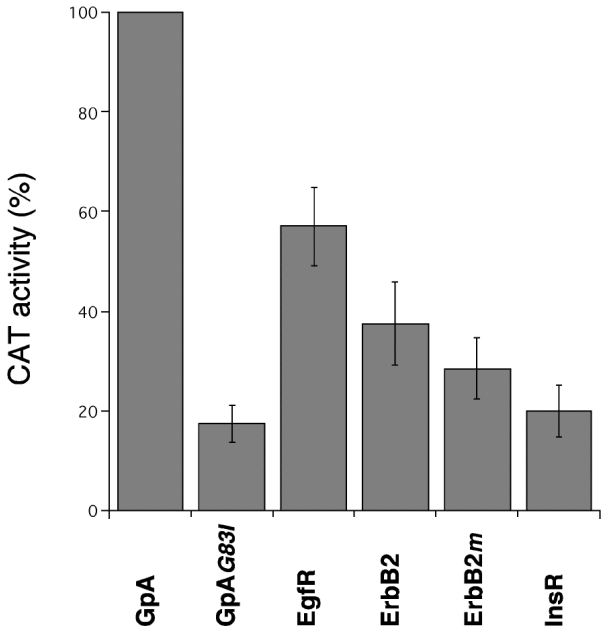


Figure 6. Homo-dimerization of the isolated receptors TM sequences. The modified TOXCAT assay TOXLUC was used to measure homo-oligomerization of the insulin, EGF, and ErbB2 receptor TM sequences in the *E. coli* inner membrane. Cells expressing the TOXCAT chimeras were lysed and assayed for luciferase activity. Activity for the glycophorin A (GpA) construct was taken as 100%. Bars report the mean ± SD values of at least three independent measurements.

sequences of an oncogenic ErbB2 mutant or glycophorin A causes constitutive activation or insulin insensitivity of this receptor, respectively [8, 9]. A possible explanation for the discrepancy with our results may reside in the lower dimerization potency of the IR TM domain. This low dimerization affinity would not allow the added peptides to interact significantly with the whole receptor in our experimental setting, but could be sufficient to contribute to the ligand induced activation within a dimeric receptor.

Another paradox is that the mutated ErbB2 sequence is a potent signaling inhibitor while displaying low dimerization potential, in fact lower than wild-type ErbB2. This last point is quite surprising, since this oncogenic Val → Glu mutation has been clearly shown to enhance dimerization and activation of full-length receptors [see e. g. ref. 4]. Using the Toxcat reporter assay, Mendrola et al. [5] also found that this mutation actually reduces the tendency of the ErbB2 TM peptide to self-associate. They proposed that the dimerization affinity of isolated TM peptides is not the only factor in their contribution to receptor activation, but that the structural details of the dimerization interface and the resulting reorientation of other receptor domains are to be considered. Replacement of the IR TM domain by the mutated ErbB2 sequence most probably imposes conformational changes which cause constitutive activation; this would then make the chimeric receptor sensitive to inhibition by exogenous correspondent peptides. Our results favor such a mechanism, but detailed confirmation will have to wait until complete crystallographic structures of full-length receptors are available.

In conclusion, the present paper shows that: (i) substitution of the IR TM domain by that of the EGFR has no effect on insulin binding, receptor autophosphorylation or the insulin effect on Akt phosphorylation, whereas the introduction of a mutant oncogenic form of the ErbB2 TM domain results in the constitutive activation of receptor kinase; (ii) the introduction of TM peptides, either through expression plasmids or as synthetic compounds, induces the inhibition of receptor activity in a specific manner, i.e. a given sequence can inhibit only the chimeric receptor containing the same TM domain, which demonstrates that TM interactions are responsible for the inhibitory effect; (iii) the IR TM peptide is ineffective, and (iv) there is a correlation between the potency of the peptide sequence and their propensity to dimerize in a bacterial hybrid system, but structural details of the dimer interface are also clearly important.

In some other receptor systems, such as the beta2-adrenergic receptor [18], the CXCR4 and CCR5 chemokine receptors [19], and the T cell antigen receptor [20], receptor function can be inhibited using hydrophobic peptides similar to their TM domains. Our results show that such a sequence-based method of disrupting membrane protein association and function is also applicable to RTKs, and may provide new insights into the mechanism underlying activation of this class of receptors.

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