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Identification of a 170 kDa Membrane Kinase With Increased Activity in KB-V1 Multidrug Resistant Cells

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Abstract Using an in situ kinase assay we have identified kinases that are elevated in some multidrug resistant cells. Kinases were detected by measurement of ^{32}P incorporation in proteins that were renatured after being subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes [Ferrell and Martin: *J Biol Chem* 264:20723–20729, 1989; *Mol Cell Biol* 10:3020–3026, 1990]. Kinases at 79, 84, and 92 kDa showed increased activity in the multidrug resistant human KB-V1 cells as compared to the sensitive parental KB-3-1 cells. The KB-V1 multidrug resistant cell line exhibited a 170 kDa membrane associated kinase activity that was not present in the parental drug sensitive line. The 170 kDa kinase activity was not affected by Ca^{++}, phosphatidylserine, or cAMP, but was diminished after incubation in the presence of the kinase inhibitors staurosporine, K252a and KT5720. The 170 kDa kinase activity phosphorylated mainly threonine, with no evidence of tyrosine phosphorylation, and was not identical to either the multidrug resistance associated P-glycoprotein or the EGF receptor. Other multidrug resistant cell lines also showed elevated 170 kDa kinase activity, such as the human breast cancer MCF-7/Adr^R and murine melanoma B16/Adr^R cells, but the activity was not present in murine leukemia P-388 sensitive or multidrug resistant cells.

Key words: multidrug resistance, kinase, 170 kDa, membrane, P-glycoprotein, EGF-receptor

Multidrug resistance of tumor cells appears to be a limiting factor in the efficacy of some types of cancer chemotherapy. Genetic selection in tumors and the consequent survival of multidrug resistant (MDR) cells is thought to account for this phenomenon [Endicott and Ling, 1989; Gottesman and Pastan, 1988]. Multidrug resistance was originally characterized in vitro after the selection of actinomycin D or colchicine resistant cells [Bech-Hansen et al., 1976; Biedler and Riehm, 1970]. These cells were shown to be resistant to a wide range of other drugs as well, such as adriamycin, vinblastine, and puromycin. The drugs to which these cells are resistant are dissimilar in both structure and mode of action. The only common features are that the drugs are hydrophobic, amphipathic compounds generally derived from natural products. Ling and co-workers showed that this multidrug resistance was correlated with the presence of a 150–170,000 molecular weight glycoprotein, which they termed P-glycoprotein [Riordan and Ling, 1985]. It is thought that the P-glycoprotein forms a molecular pump, which actively causes the excretion or efflux of drugs from the cell membrane or cytoplasm [Endicott and Ling, 1989; Gottesman and Pastan, 1988; Horio et al., 1988]. While the P-glycoprotein was originally studied in rodent cells, elevated levels have now been found in human cells that have been selected for drug resistance in vitro, as well as in multidrug resistant cancers [Goldstein et al., 1989].

While increased amounts of P-glycoprotein appear to be important for the development of some cases of multidrug resistance, it does not appear to be the only mechanism by which cells

Abbreviations used: MDR, multidrug resistant; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene difluoride; BSA, bovine serum albumin; EGF, epidermal growth factor; PBS, Dulbecco's phosphate-buffered saline; TN, 10 mM Tris-HCl, pH 7.4, 0.5 M NaCl; PMA, phorbol 12-myristate 13-acetate.

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can become multidrug resistant. Several workers have reported that cells selected for multidrug resistance show evidence of non-P-glycoprotein mediated multidrug resistance mechanisms [Beck et al., 1987; Cole et al., 1991; Shen et al., 1991] and multidrug resistant cells may exhibit more than one resistance mechanism simultaneously [Zijlstra et al., 1987].

There is also accumulating evidence that protein kinases may be involved in the expression and function of the P-glycoprotein and multidrug resistance. Several studies have described an increase in kinase activities or increased phosphorylation of the P-glycoprotein in multidrug resistant cells [Carlsen et al., 1977; Center, 1983, 1985; Fine et al., 1988; Hamada et al., 1987; Mellado and Horwitz, 1987; Posada et al., 1989]. Higher levels of protein kinase C are associated with increased multidrug resistance of some but not all multidrug resistant cells [Chambers et al., 1990; Fine et al., 1988; Posada et al., 1989]. Yu et al. [1991] have shown that transfection of a plasmid expressing functional protein kinase Cα is associated with an increase in multidrug resistance. Our laboratory has shown that cAMP-dependent protein kinase levels are directly associated with relative multidrug resistance [Abraham et al., 1987] and P-glycoprotein mRNA and protein levels in Chinese hamster ovary cells [Abraham et al., 1990]. These studies suggest that protein kinases may have a role in modulating multidrug resistance.

We have used an in situ kinase assay developed by Ferrell and Martin [1989, 1990] to examine kinase activity in multidrug resistant cells. We hoped to identify both known and unknown kinases active in this assay, define their apparent molecular weight, and determine any specific changes in kinase activity in multidrug resistant cells as compared to the drug sensitive parent cell lines. Phosphorylated bands identified by this technique represent kinase autophosphorylation, phosphorylation of a cellular substrate whose migration is identical to the kinase, or phosphorylation of an exogenous substrate from the blocking agent in the in situ reaction [Ferrell and Martin, 1990]. Phosphorylation will not be apparent on proteins that are already fully phosphorylated. Our evidence shows an increase in the activity levels of several kinases in multidrug resistant cells. We have identified a 170 kDa membrane associated protein kinase activity in the human carcinoma KB-V1 multidrug resistant cells which was not seen in the parent KB-3-1 cells. Other cells have also been found to express this activity, with a significantly greater level of activity in multidrug resistant cells than in the parent drug sensitive cells. This suggests a possible role for this kinase in multidrug resistance, although neither the exact function of this kinase nor its substrates have been identified.

METHODS

Materials

Protein kinase inhibitors staurosporine, K252a, and KT5720 were obtained from Kamiya Biomedical Co. (Thousand Oaks, CA), and inhibitor H-7 was from Seikagaku America, Inc. (St. Petersburg, FL). Vinblastine and colchicine were from Sigma Chemical Company (St. Louis, MO). Murine monoclonal anti-phosphotyrosine antibody kit was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Murine monoclonal P-glycoprotein antibody C219 was from Centocor Diagnostics (Malvern, PA). Phosphatidylserine was purchased from Serdary Research Laboratories (London, Ontario, Canada).

Cell Culture

The human carcinoma cell line KB-3-1 and the multidrug resistant cell line KB-V1 [Shen et al., 1986] were obtained from M. M. Gottesman (NIH, Bethesda, Md). The cells were grown in DME with high glucose (Irvine Scientific, Santa Ana, CA) containing 10% FBS (Gibco BRL, Gaithersburg, MD), 110 μg/ml sodium pyruvate, 4 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The KB-V1 line was maintained with the addition of 1 μg/ml vinblastine. Human A431 cells were obtained from the American Type Culture Collection (CRL 1555). LMTK$^-$ E-KK cells (EPVI-2A1-V180) are murine LMTK$^-$ cells transfected with a plasmid containing both the wildtype human MDR1 cDNA and a gene conferring resistance to Geneticin, obtained from I. Roninson, University of Illinois [Morse and Roninson, 1990]. The MDR1 gene codes for P-glycoprotein and confers multidrug resistance. E-KK cells were grown in the presence of 0.4 ng/ml Geneticin (Gibco BRL) and 110 nM vinblastine. KB-GRC1 cells are KB cells transfected with the wildtype human MDR1 gene [Choi et al., 1988], obtained from D. Shalinsky (University of California, San Diego), and grown in the presence of 6 ng/ml colchicine. A431, LMTK$^-$, and KB-GRC1 cells were grown

in media described above for KB cells. Mouse P388 and P388/Adr^R cells [Shabel et al., 1983] were received from L. Li (Upjohn) and grown in RPMI 1640, 5% heat inactivated FBS, 25 mM Hepes, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin. Human MCF7 and MCF7/Adr^R [Batist et al., 1986] cells from L. Li were grown in P388 media with 10% heat inactivated FBS. Murine B16/BL6 and B16/Adr^R [Sebolt et al., 1989] cells from L. Li were grown in RPMI 1640 with 10% FBS, 50 μg/ml gentamicin.

In Situ Kinase Assay

The procedure was followed as previously published [Ferrell and Martin, 1989, 1990] with minor modifications. Cells were lysed in SDS sample buffer and subjected to SDS-polyacrylamide electrophoresis [Laemmli, 1970] on an 8% (or 6% as noted) gel. Proteins were immediately transferred to Immobilon PVDF membrane (Millipore, Bedford, MA) denatured in guanidine HCl and renatured as previously described. After blocking for 1 h in 5% BSA, blots were treated with 30 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, and 25 μCi/ml [γ-^{32}P]ATP (6000 Ci/mmol, Amersham, Arlington Heights, IL) for 30 min at room temperature. The kinase reaction was terminated by washing repeatedly in 30 mM Tris-HCl, pH 7.4, followed by a 10 min treatment with 1 M KOH to reduce background. The KOH wash was occasionally deleted and did not alter the results. The blots were neutralized with 10% acetic acid and H_2O washes, then exposed to Kodak X-Omat AR film at −80°C with intensifying screens.

In some experiments, protein blots were cut into strips prior to the kinase reaction, and the strips placed in individual chambers. Agents were added to the kinase reaction solution in each chamber at indicated concentrations, and the kinase reactions then proceeded for 30 min at room temperature followed by washing as normal.

Membrane Fraction Isolation

Cells were suspended in 10 mM Tris-HCl, pH 7.4, 250 mM sucrose and 10 μg/ml aprotinin and leupeptin, then lysed by sonication using a Vibracell (Sonics and Materials, Inc., Danbury, CT). Samples were centrifuged at 100,000*g* at 4°C for 30 min, the cytosolic supernatant was removed, and the membrane fraction pellet was resuspended in the Tris-sucrose buffer. Samples of 50 μg protein were loaded per lane on SDS-polyacrylamide gels, without heat denaturation. Proteins were transferred to Immobilon PVDF membrane and the kinase reaction was carried out as above.

Anti-Phosphotyrosine Immunoblot

A431 cells were treated with 100 ng/ml recombinant human EGF (Gibco BRL) for 1 h at 37°C to activate the autophosphorylation of the EGF receptor. Treated or untreated (control) cells were then harvested in Tris-sucrose buffer with the addition of 1 mM sodium orthovanodate, sonicated and centrifuged as described above to yield a membrane fraction. Samples were subjected to PAGE on a 6% polyacrylamide gel, and proteins were transferred to Immobilon PVDF membrane followed by the in situ kinase assay. After autoradiograph analysis of kinase activity, PVDF protein blots were incubated overnight at 4°C with murine monoclonal anti-phosphotyrosine antibody diluted to 1 μg/ml in 3% non-fat dry milk in PBS (Gibco BRL). After rinsing in PBS, the blot was treated for 1 hr with goat anti-mouse IgG alkaline phosphatase conjugate diluted in the same buffer, then washed in PBS with 0.05% Tween-20. The blot was rinsed in PBS 4–5 times, then developed in alkaline phosphatase substrate solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate disodium until colored bands were visible.

P-Glycoprotein Immunoblot

Membrane protein samples of 50 μg per lane were subjected to electrophoresis on SDS-polyacrylamide gels and transblotted to nitrocellulose membrane in 25 mM Tris, 192 mM glycine containing 20% methanol. Blots were blocked in 3% non-fat dry milk in PBS, then incubated overnight at 4°C with the mouse monoclonal anti-P-glycoprotein antibody, C219, diluted to 0.5 μg/ml in 10 mM Tris-HCl, pH 7.4, 0.5 M NaCl (TN) with 3% BSA. The blot was washed in TN, then treated with rabbit anti-mouse IgG in TN with 3% BSA for 1 h at room temperature. The blot was washed again, treated for 1 h with [^{125}I]Protein A (NEN DuPont, Boston, MA) at 0.1 μCi/ml in TN with 3% BSA, then washed before being exposed to Kodak X-Omat AR film at −80°C with intensifying screens.

In Vitro Phosphorylation

Phosphorylation reactions proceeded according to the protocol of Akiyama et al. [1987]. Each sample reaction contained 20 mM Pipes, pH 7.2, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM dithiothrietol, 100 mM sodium orthovanadate, 10 μM [γ-^{32}P]ATP at 20 Ci/mmol, and 10 μg of cell membrane protein, in a final volume of 50 μl. EGF receptor phosphorylation reactions included 1 μg/ml recombinant human EGF and proceeded at 4°C for 5 min. General kinase activity reactions were performed without EGF at 30°C for 5 min. Reactions were terminated by addition of SDS sample buffer and boiling for 5 min. Samples were subjected to PAGE on a 6% polyacrylamide gel and analyzed by autoradiography.

Phosphoamino Acid Analysis

Phosphoamino acid analysis was performed by the method of McCroskey et al. [1988]. PVDF-bound ^{32}P-labeled phosphoproteins were visualized by autoradiography after the in situ kinase reaction as described above except with the deletion of BSA from the renaturation buffer and the elimination of the BSA blocking buffer incubation. The initial 10 min, 1 M KOH wash of the PVDF membrane described above was occasionally deleted to prevent phosphoserine or -threonine degradation, but this did not change the overall phosphorylation pattern. Portions of the membrane corresponding to the 170 kDa ^{32}P-labeled band were directly hydrolyzed [Kamps and Sefton, 1989; McCroskey et al., 1988] for either 1 or 3 h in 6 N HCl at 110°C under N_2. The HCl-hydrolysate was removed, placed in a 1.5 ml Eppendorf microtube and dried in a rotary evaporator. The residue was resuspended in 35 μl of 35 mM phosphoric acid, brought to pH 3.2 by addition of 10 N KOH. The sample was then injected onto a Vydac oligonucleotide anion exchange HPLC column (#304OL54, The Separations Group, Hesperia, CA) equilibrated with 35 mM phosphate buffer, pH 3.2. Phosphoamino acids were eluted from the column isocratically at a flow rate of 0.6 ml/min. One minute fractions were collected and quantitated by Cerenkov counting. Phosphoamino acids were identified by comparison of retention time of ^{32}P-labeled peaks with those of phosphoamino acid standards monitored at 206 nm.

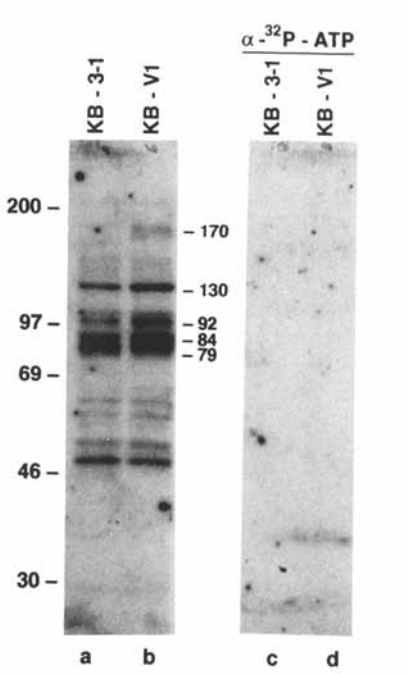

Fig. 1. Increased kinase activity in multidrug resistant cells Autoradiographs of phosphorylated proteins on PVDF blots after 100 μg protein from whole cell lysate of KB-3-1 cells (**lanes a,c**) or KB-V1 cells (**lanes b,d**) was run on SDS-polyacrylamide electrophoresis, blotted, and treated for the in situ kinase assay as in Methods Molecular weight markers are indicated on the left in kilodaltons Lanes c and d show the in situ reaction substituting [α-^{32}P] ATP for the [γ-^{32}P] ATP

RESULTS

Multidrug resistant cells (KB-V1) and their parental drug sensitive cells (KB-3-1) were compared for the presence of kinase activity using an in situ assay developed by Ferrell and Martin [1989, 1990]. Cell lysate proteins were subjected to electrophoresis on SDS-polyacrylamide gels, blotted to PVDF membranes and denatured in guanidine hydrochloride. The protein blot was then renatured overnight followed by incubation in [γ-^{32}P]ATP, allowing transfer of phosphoryl residues by the immobilized kinases. Autoradiograph analysis revealed the radiolabeled bands on the blot which corresponded to the position of the active kinases. Several kinase activities were increased in the multidrug resistant KB-V1 cells as compared to the parental KB-3-1 cells, with phosphorylated bands at 79, 84, 92, and 130 kDa strongly increased (Fig. 1). The 79 kDa kinase comigrates with protein kinase C (data not shown). A 170 kDa band was seen only in the multidrug resistant KB-V1 cells and not in the sensitive KB-3-1 cells, suggesting a connection between the presence of this kinase

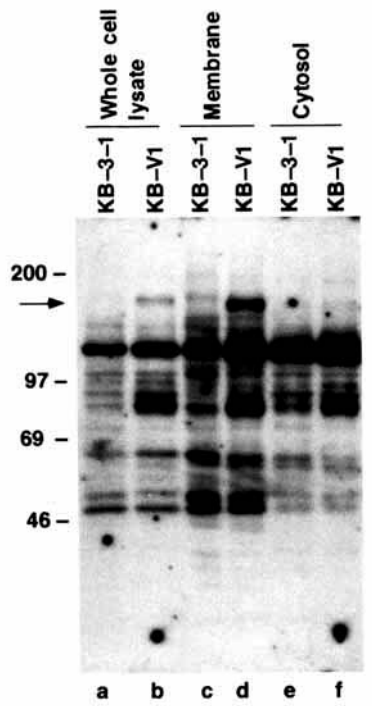

Fig. 2. Localization of 170 kDa kinase in the membrane fraction. Whole cell lysates, membrane, and cytosol were isolated as described in Methods. 100 μg protein of whole cell lysate of KB-3-1 **(lane a)** or KB-V1 **(lane b)** or 50 μg protein of membrane of KB-3-1 **(lane c)** or KB-V1 **(lane d)** or 50 μg protein of cytosol of KB-3-1 **(lane e)** or KB-V1 **(lane f)** was subjected to SDS-polyacrylamide electrophoresis, then blotted and treated in the in situ assay. The arrow indicates 170 kDa.

activity and multidrug resistance. Because of the possibility of the radioactive bands reflecting ATP binding rather than covalent transfer of phosphate, [α-^{32}P] ATP was substituted for [γ-^{32}P]ATP. As shown in Figure 1, lanes c and d, no bands were seen on the autoradiograph after the in situ kinase reaction. This confirms that the bands seen in lanes a and b were due to γ-phosphate transfer and not bound ATP.

Activity of the 170 kDa kinase was originally seen in whole cell lysates. Upon cell fractionation, it was apparent that this kinase was most abundant in the membrane fraction of KB-V1 cells (Fig. 2). The 84 and 92 kDa kinases were found mainly in the cytosol of both KB-3-1 and KB-V1 cells (lanes e and f). There was some evidence of presence of the 84 kDa and 92 kDa kinase in the KB-V1 membrane fraction as well. The 79 and 130 kDa kinases were found in membrane and cytosol fractions of both cells (lanes c–f). The 130 kDa kinase was not always present; the reason for this is not clear. Variation in the kinase activities in KB-V1 cells was seen in different experiments, primarily with the 130 and the 79 kDa kinase activity (compare Fig. 1 to Fig. 2), but the activity for the kinases was generally greater than that seen with the KB-3-1 cells. We concentrated our attention on the 170 kDa activity due to its consistency of activity in the drug resistant KB-V1 cells, and its large size.

We tested the effect of various kinase activators and inhibitors when added to the in situ kinase assay reaction mixture (Fig. 3). Since a kinase reported to have high activity in HL60 multidrug resistant cells has been shown to be activated in the presence of phosphatidylserine or phosphatidylinositol in a conventional kinase assay [Staats et al., 1990], we tested whether any of the kinases detected by the in situ assay were stimulated under such conditions. None of the kinases, including the 170 kDa kinase activity, were enhanced by phosphatidylserine, Ca^{++}, or both. It is possible, however, that conditions in the in situ assay might not be sufficient to show phosphatidylserine dependence of kinase activity. To further define the characteristics of the kinases, we also examined changes in activities after incubation of the renatured blots with various protein kinase inhibitors included in the in situ kinase reaction (Fig. 3). All the inhibitors used have a broad selectivity and will inhibit multiple kinases, but staurosporine has been reported to have a high specificity for inhibition of protein kinase C, while KT5720 is most specific for cAMP-dependent protein kinase inhibition. Staurosporine and K252a inhibited most of the kinase activities to some extent, but appeared ineffective in inhibiting the 130 kDa kinase. KT5720 was effective in inhibiting the 170, 84, and 79 kDa kinase activities. H-7, a low potency inhibitor of cAMP-dependent protein kinase and protein kinase C, did not appear to inhibit any of the kinases with increased activity in the KB-V1 cells when tested at 20 μM.

In order to determine the divalent cation requirements of the 170 kDa kinase, a series of concentrations of Mg^{++} and Mn^{++} were tested (Fig. 4). The standard kinase reaction included 10 mM Mg^{++} and 2 mM Mn^{++} (lane i); however, the optimal 170 kDa kinase activity was seen at 10 mM Mn^{++} in the absence of Mg^{++} (lane c).

It has been reported that the EGF receptor, known to have tyrosine kinase activity, is elevated in some multidrug resistant cells [Meyers et al., 1986, 1988]. The EGF receptor has been reported to appear between 170 and 175 kDa molecular weight [Cohen, 1983; Waterfield et

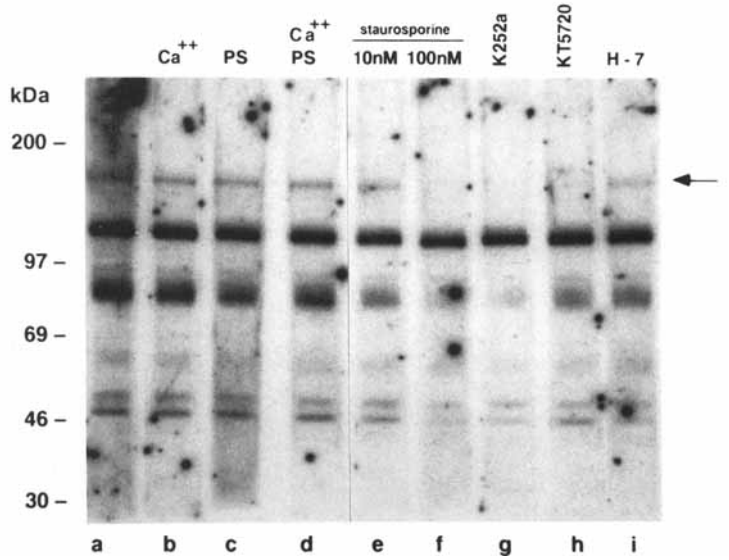

Fig. 3. Sensitivity of 170 kDa kinase to kinase activators or inhibitors added to the in situ reaction Protein blots were cut into strips prior to the kinase reaction, then placed in individual chambers in kinase reaction buffer along with a series of agents as indicated in the figure The kinase reaction proceeded for 30 min and then the blots were washed as normal **Lane a,** control, **lane b,** 200 μM Ca^{++}, **lane c,** 100 μg/ml phosphatidylserine, **lane d,** 200 μM Ca^{++} + 100 μg/ml phosphatidylserine, **lane e,** 10 nM staurosporine, **lane f,** 100 nM staurosporine, **lane g,** 0 5 μM K252a, **lane h,** 5 μM KT5720, **lane i,** 20 μM H-7 Arrow indicates 170 kDa

al., 1982] on SDS-polyacrylamide gels. Because of the similarity in molecular weight, we investigated whether the EGF receptor was identical to the 170 kDa kinase. Immunoprecipitation with anti-EGF receptor monoclonal antibody resulted in the identification of the EGF receptor in A431, KB-3-1, and KB-V1 cells at 175 kDa molecular weight, clearly higher in molecular weight than the 170 kDa kinase (data not shown). In addition, we were able to differenti-

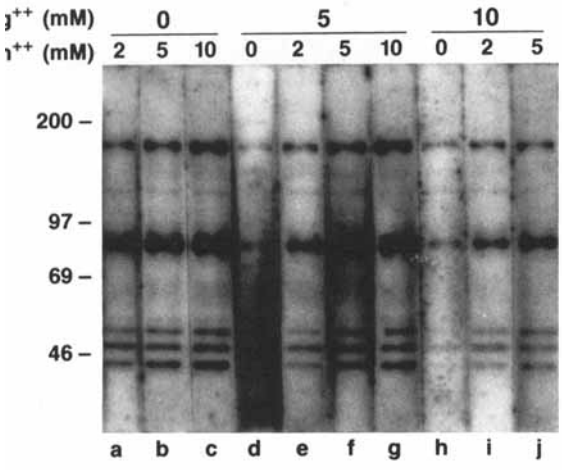

Fig. 4. Effect of divalent cations on 170 kDa kinase activity Protein blots were cut into strips as in Figure 3 and tested in the kinase reaction containing different concentrations of Mg^{++} and Mn^{++} as indicated in the figure

ate between the EGF receptor and a 170 kDa band in an in vitro membrane phosphorylation assay using a previously described method [Akiyama et al., 1987]. Incubating the reaction mix containing EGF at 4°C for 5 min resulted in phosphorylation of the EGF receptor and no other kinases (Fig. 5, lane a). When the reaction was carried out without EGF at 30°C for 5 min, both the EGF receptor and a phosphorylated band at 170 kDa were seen, as well as several lower molecular weight phosphorylated bands. Since there is no P-glycoprotein expressed in A431 cells (not shown), this 170 kDa phosphorylated protein probably corresponds to the 170 kDa kinase seen in the in situ assay.

We also investigated whether the 170 kDa kinase activity was that of a tyrosine kinase. Anti-phosphotyrosine monoclonal antibody recognized the autophosphorylated EGF receptor as a 175 kDa band on a Western blot of EGF-treated A431 cells (Fig. 6A, lane b), but did not recognize the 170 kDa kinase phosphorylated band from untreated A431 cells (lane a), or from KB-V1 cells (not shown). The anti-phosphotyrosine reactive band was clearly larger than the 170 kDa kinase from the A431 and KB-V1 cells (Fig. 6B). It also appeared that the EGF receptor was not active in the in situ kinase reaction. This was consistent with previous findings which dem-

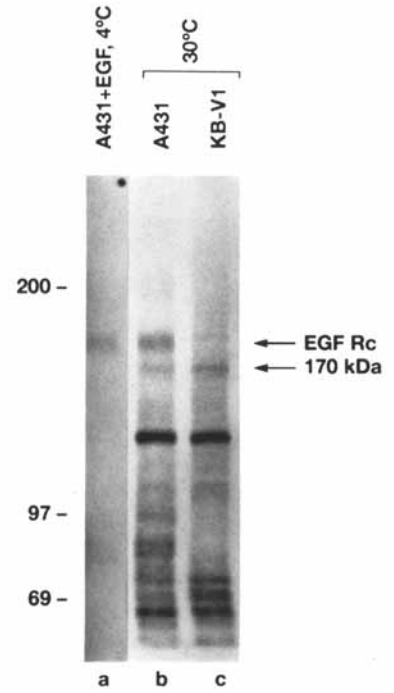

Fig. 5. The EGF receptor and the 170 kDa kinase phosphorylated in vitro A431 or KB-V1 membrane proteins were used in an in vitro phosphorylation to compare the EGF receptor (EGF Rc) to the 170 kDa kinase **Lane a:** A431 membrane protein treated with EGF at 4°C **Lane b:** A431 membrane protein **Lane c:** KB-V1 membrane protein Lanes b and c contained no EGF, and phosphorylation proceeded at 30°C, as in Methods Arrows on the right show position of the EGF Rc and the 170 kDa kinase

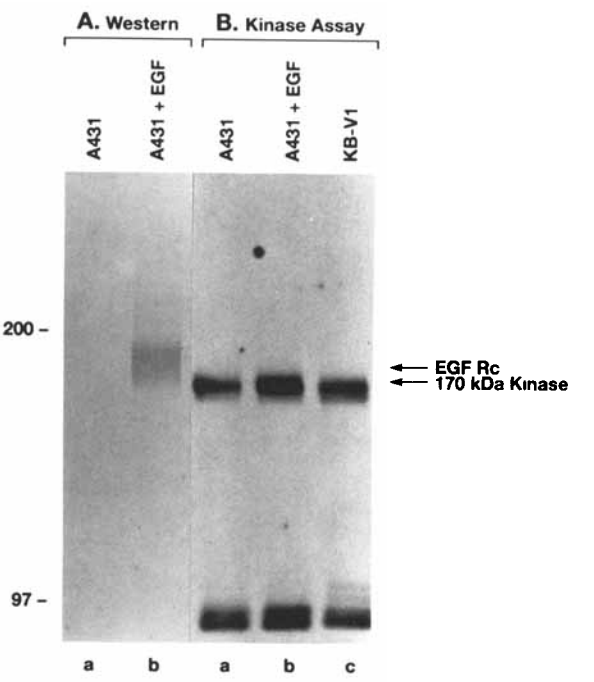

Fig. 6. 170 kDa kinase is not the EGF receptor and does not show tyrosine kinase activity A431 and KB-V1 cells were untreated or treated with 100 ng/ml EGF for 1 h prior to harvest Cell membrane fractions were analyzed for 170 kDa kinase activity, after electrophoresis and blotting from a 6% gel, detected by autoradiography **(B)**, and then for phosphotyrosine antibody reactivity, detected by color reaction **(A)**, on the same PVDF blot **Lane a:** A431 membrane protein **Lane b:** EGF-treated A431 membrane protein **Lane c:** KB-V1 membrane protein

onstrated the absence of tyrosine kinase activities in the in situ kinase assay [Ferrell and Martin, 1989]. The 170 kDa band intensity was significantly reduced after prolonged washing of the ^{32}P-labeled PVDF blot with 1 M KOH at 55°C (data not shown). This also suggested that the 170 kDa kinase was not a tyrosine kinase since tyrosine-phosphate bonds are relatively resistant to treatment with strong alkali [Cooper and Hunter, 1981]. Finally, phosphoamino acid analysis was performed by hydrolyzing proteins present in the region of the 170 kDa kinase and fractionating the hydrolysate on an oligonucleotide anion exchange HPLC column (Fig. 7). There was no evidence of any ^{32}P-labeled phosphotyrosine present in the region of the 170 kDa kinase. Most of the labelled phosphoamino acid was in the form of phosphothreonine, with a smaller amount of phosphoserine apparent. These results, together with the 170 kDa kinase inhibition by inhibitors of serine/threonine kinases seen in Figure 4A, suggested that the 170 kDa kinase is most probably a serine/threonine kinase. The lack of tyrosine kinase activity at the position of the 170 kDa activity, the inability to be recognized by anti-phosphotyrosine antibody, and the difference in size from the EGF receptor on Western immunoblots clearly showed that the 170 kDa kinase is not the EGF receptor.

Several other sensitive and drug resistant cell lines were examined for 170 kDa kinase activity by in situ assay (Fig. 8A). The mouse melanoma multidrug resistant cell line B16/Adr^{R} showed a significant increase in 170 kDa kinase activity as compared to the parent drug sensitive B16/BL6 cells. The 170 kDa kinase activity was also increased in human breast cancer multidrug resistant cell line MCF7/Adr^{R} as compared to the parent MCF7 cells (data not shown). Neither the mouse leukemic P388 cells nor their multidrug resistant subline P388/Adr^{R} expressed the 170 kDa kinase, indicating that the kinase is not always present in multidrug resistant cell lines. All these cell lines were also tested for expression of the multidrug resistance-associated P-glycoprotein. A Western blot was reacted with the

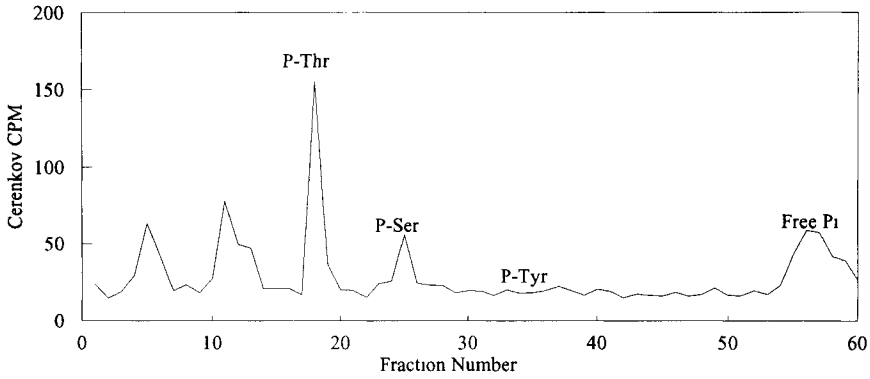

Fig. 7. Phosphoamino acid analysis of 170 kDa phosphoprotein Phosphoamino acid analysis of the 170 kDa protein was performed on an oligonucleotide HPLC column as described in Methods Hydrolysis was carried out for 3 h Phosphoamino acids identified by comparison with standards are indicated

monoclonal antibody C219, known to react with murine, human, and hamster P-glycoprotein, and the resulting autoradiograph (Fig. 8B) showed that all the multidrug resistance cells tested (including MCF7/Adr^R, not shown) express the P-glycoprotein at between 150 and 180 kDa. Presence of the 170 kDa kinase in cells that did not express P-glycoprotein suggested that they are separate molecules. However, the sensitivities of the Western blot for P-glycoprotein and the kinase assay are probably different, and it is possible that we did not detect very low levels of P-glycoprotein. The differences in molecular weight of the P-glycoprotein and the 170 kDa kinase in some cell lines also confirmed that they are separate proteins, and that the P-glycoprotein is not the substrate of the immobilized 170 kDa kinase in the in situ assay.

In order to further exclude the possibility that the 170 kDa kinase activity was due to P-glycoprotein, we examined cells that were transfected with MDR1, the human P-glycoprotein gene (Fig. 9). Transfected KB cells (KB-GRC1) and mouse LMTK$^-$ cells (E-KK cells) were used in order to determine if the gene for the 170 kDa kinase was identical to the gene for P-glycoprotein. These cells had previously been shown to exhibit the multidrug resistance phenotype [Choi

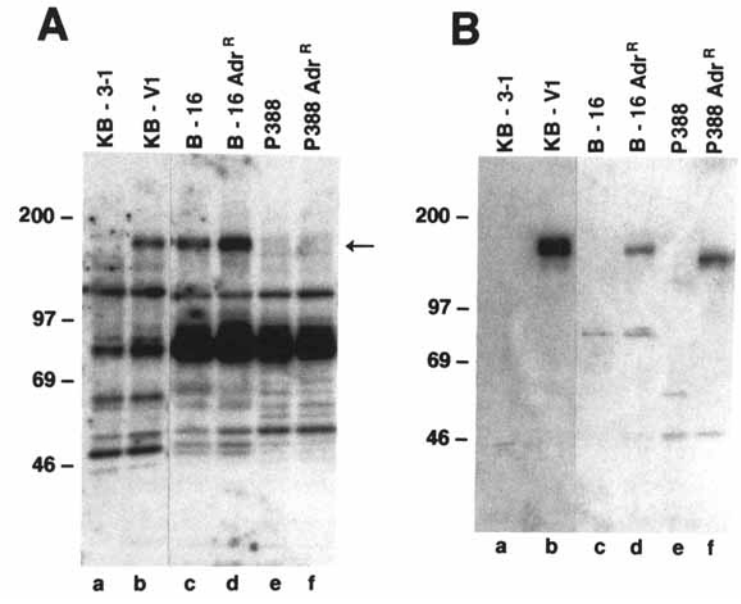

Fig. 8. Presence of 170 kDa kinase in various cell lines with and without P-glycoprotein **A:** Drug sensitive cells KB-3-1, B16/BL6, P388 (**lanes a,c,e,** respectively) and multidrug resistant cells KB-V1, B16/Adr^R, P388/Adr^R (**lanes b,d,f,** respectively) were tested in the in situ kinase assay 170 kDa kinase is indicated by the arrow **B:** Western blot analysis of P-glycoprotein expression, using the C219 monoclonal antibody specific for the P-glycoprotein

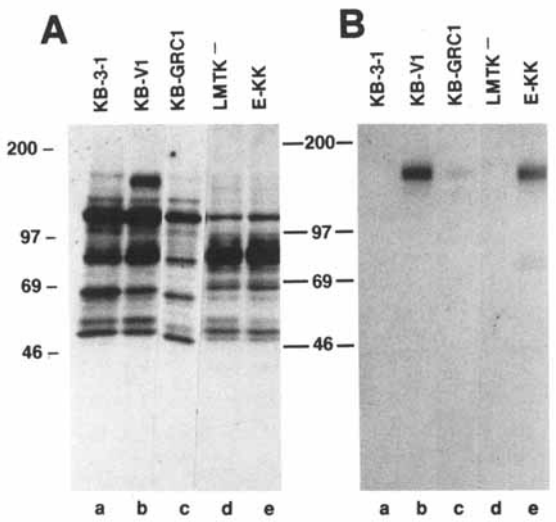

Fig. 9. Absence of kinase activity in MDR1-transfected cells Mouse LMTK cells and human KB-3-1 cells had been transfected with the normal human MDR1 gene encoding the P-glycoprotein and conferring multidrug resistance [Choi et al., 1988, Morse and Robinson, 1990] **A:** 170 kDa kinase activity measured by in situ kinase assay in KB-3-1, KB-V1, KB-GRC1, (MDR1 transfectant), LMTK⁻ and LMTK E-KK (MDR1 transfectant), (lanes **a–e**, respectively) **B:** Expression of the P-glycoprotein determined by Western blot as in Figure 8B in KB-3-1, KB-V1, KB-GRC1, LMTK , and LMTK E-KK, (lanes **a–e**, respectively)

et al., 1988; Morse and Ronison, 1990]. Western blot analysis using the C219 antibody showed that all the transfectant cells expressed the P-glycoprotein, although expression in the KB-GRC1 cells was low (Fig. 9B). The in situ kinase assay showed that none of the transfectants contained the 170 kDa kinase activity (Fig. 9A). This corroborates our finding that the P-glycoprotein and the 170 kinase activity are different proteins, and that the 170 kDa kinase activity is not invariably present in cells expressing the P-glycoprotein.

DISCUSSION

Prior studies have indicated that increases in kinase activity or quantity are associated with multidrug resistance, and have suggested a role for kinases in the regulation of the function or levels of the P-glycoprotein [Abraham et al., 1990; Center, 1985; Chambers et al., 1990; Fine et al., 1988; Hamada et al., 1987; Mellado and Horwitz, 1987; Posada et al., 1989; Yu et al., 1991]. Regulation may occur by changes in the amount of phosphorylation of the P-glycoprotein molecule leading to activity changes [Center, 1985; Chambers et al., 1990; Hamada et al., 1987; Posada et al., 1989] or through control of levels of P-glycoprotein mRNA or protein [Abraham et al., 1990]. We have recently begun a search for altered kinase activities in multidrug resistant cells, using the in situ kinase assay developed by Ferrell and Martin [1989, 1990]. These kinases may modulate P-glycoprotein function, or may affect unknown substrates that alter cellular resistance.

Several kinases were identified in cell lysates of human carcinoma KB-3-1 cells. Approximately 12 radiolabelled bands of varying intensity, ranging in size from 45 kDa to 150 kDa, were clearly identified on the autoradiograph. Of these, 4 bands at 79, 84, 94, and 130 kDa showed an obvious increase in intensity in the multidrug resistant KB-V1 cell line, although there was some variability in their detection from assay to assay. The change in intensity of a band may indicate an altered amount of the kinase or of a substrate at the same position on the blot. It may also reflect a change in the activity of the kinase, resulting in a change in the level of autophosphorylation or phosphorylation of a substrate. Coomassie staining of SDS-polyacrylamide gels did not show any significant changes in protein content between the KB-3-1 and the KB-V1 cells (not shown). This finding was not definitive since these kinases may not be present in sufficient amounts to be detected by Coomassie staining. In addition to the 79, 84, 94, and 130 kDa bands, a band at 170 kDa was seen in multidrug resistant KB-V1 cells only. Its presence only in the multidrug resistant cells suggested a function connected with drug resistance, and its similarity in size to the P-glycoprotein led us to characterize this kinase further. The 170 kDa kinase is membrane-associated and has optimal activity in 10 mM Mn^{++}. It was not evident from our data whether the substrate of the 170 kDa kinase in this assay is the enzyme itself, or a protein overlaying it on the blot.

Previous reports have shown that the EGF receptor is elevated in some multidrug resistant cells [Meyers et al., 1986, 1988]. Since the EGF receptor is similar in size to 170 kDa kinase, we investigated whether it was identical to the 170 kDa kinase. The 170 kDa kinase was established to be smaller than the EGF receptor in A431 and KB cells after comparing the molecular weights of the 170 kDa kinase identified on the in situ activity blots and the EGF receptor identified either by anti-phosphotyrosine Westerns or immunoprecipitation with an anti-EGF receptor antibody.

The fact that the 170 kDa kinase was not recognized by anti-phosphotyrosine monoclonal

antibody suggests that it is not a tyrosine kinase. Other experiments showed that the radiolabelled phosphate residue(s) of the 170 kDa band was easily removed by prolonged KOH treatment, also suggesting that it is not a tyrosine kinase. In addition, phosphoamino acid analysis of proteins eluted from the PVDF membrane in the area of the 170 kDa kinase did not show any evidence of phosphotyrosine. The presence of phosphothreonine and some phosphoserine in the phosphoamino acid digests suggested instead that the 170 kDa kinase is a serine-threonine kinase. This result also supported the evidence that the 170 kDa kinase is not P-glycoprotein, since P-glycoprotein appears to be phosphorylated mainly on serine residues [Hamada et al., 1987]. This kinase probably has structural similarities to other known serine-threonine kinases since it is inhibited by the serine-threonine kinase inhibitors staurosporine, K252a, and KT5720. However, the large size of this kinase makes it unusual, since most identified serine-threonine kinases are less than 100 kDa in size.

In a study using the in situ kinase assay, Ferrell and Martin [1990] identified a 170 kDa kinase (PK170) in whole cell lysates of human platelets. Activity of this kinase as demonstrated in the in situ assay was increased when platelets were pretreated in vivo with thrombin or both phorbol 12-myristate 13-acetate (PMA) and ionomycin. They postulated that this increase in activity might be due to a change in the phosphorylation state of the kinase. The 170 kDa kinase described here showed no stimulation when KB-V1 cells were pretreated with PMA or ionomycin, in combination or separately (not shown). This suggests that the 170 kDa kinase described here may be distinct from the platelet-derived PK170 kinase. However, signalling mechanisms present in the platelet cell that affect PK170 may not be present in the KB-V1 cells that were investigated here. Interestingly, the PK170 and the 170 kDa kinase described here both appeared to cause phosphorylation principally on threonine. The 170 kDa activity described here was found associated exclusively with the membranes. The large size and membrane localization of the 170 kDa kinase suggests that it may have some type of receptor function. A new class of membrane-bound receptors with serine-threonine kinase activity that recognizes ligands in the TGF family has recently been identified [Massagué, 1992]. It is possible that this kinase plays a similar role.

Staats et al. [1990] described a membrane associated phospholipid-stimulated kinase, found in both drug sensitive HL60 and in multidrug resistant HL60/Vinc cells which is capable of phosphorylating the P-glycoprotein in vitro. The 170 kDa kinase activity described here, however, was found only in the drug resistant KB-V1 cells and not in the parental sensitive KB-3-1 cells, although it was found in some other sensitive cells. The 170 kDa kinase studied here showed optimal activity with Mn^{++}, similar to the enzyme described by Staats et al. [1990], but unlike that enzyme, was not stimulated by phosphatidylserine. It is possible, however, that activation by phosphatidylserine might not occur in the in situ assay since kinases were presumably no longer associated with membrane. Significantly, however, membrane fractions of HL60 cells used in the in situ assay showed no 170 kDa kinase activity (data not shown). Staats et al. [1990] suggested that the HL60 kinase was similar to protein kinase P, a platelet kinase that is described by Elias and Davis [1985] as 27 kDa. These results suggest that the 170 kDa kinase is different from the described HL60 kinase.

In addition to KB-V1, other multidrug resistant cell lines were examined for 170 kDa kinase activity by in situ assay. The 170 kDa kinase was not seen in all multidrug resistant cell lines; for example, it was not present in P388/Adr^R. The presence of 170 kDa kinase activity, therefore, does not always correlate with expression of multidrug resistance. However, in the cell lines which did express the 170 kDa kinase, the activity in the multidrug resistant cells was always higher than that in the corresponding drug sensitive parent cells. One explanation of these results is that an increased activity level of the 170 kDa kinase in resistant cells is more likely if it is already detectable in the parental sensitive cells. We also saw expression in A431 cells, but had no drug resistant line with which to compare. This evidence suggests that this protein kinase may be associated with the multidrug resistance phenotype in general and with the increased expression and activity of the P-glycoprotein in particular in some multidrug resistant cells. However, since it is likely that the kinase assay is more sensitive than the Western assay for P-glycoprotein, we can not exclude the possibility that cells such as A431 may express some undetected P-glycoprotein. An increase in 170 kDa kinase activity may be independently selected in some multidrug resistant cells. Alternatively, 170 kDa kinase activity could be con-

trolled or co-amplified in cells along with the amplified P-glycoprotein.

Because P-glycoprotein has been reported to have a molecular weight of 170 kDa in KB-V1 cells, we performed experiments to determine if it was a distinct molecule from the 170 kDa kinase. All the tested multidrug resistant cells were shown to express P-glycoprotein by Western blot analysis. The molecular weight of the P-glycoprotein varied from 150 to 180 kDa in these cell lines, possibly due to differences in glycosylation. In comparison, the 170 kDa kinase molecular weight was invariant as could be seen by inspection of the autoradiographs. Because the 170 kDa kinase activity was seen in some cells which do not express the P-glycoprotein, and because of the difference in molecular weight of the P-glycoprotein in some cell lines, we conclude that the 170 kDa kinase is a separate protein from P-glycoprotein.

Cells transfected with the MDR1 gene and selected for vinblastine resistance did express the P-glycoprotein, but did not have 170 kDa kinase activity. The lack of the 170 kDa kinase in the transfected cells definitely shows that this kinase activity is not due to P-glycoprotein. The drug resistant KB-V1 cells were mutagenized and then selected for multidrug resistance by growth in vinblastine over many generations, and their resistance is thought to be due in large part to the amplification of the endogenous gene encoding the P-glycoprotein. During the selection process, however, other changes to the genome of the cell may have occurred. The KB-GRC1 cells, on the other hand, underwent a shorter selection time. The difference in origin of the drug resistant KB-V1 cells as compared to the KB-GRC1 transfectants may explain the high level of 170 kDa kinase in the KB-V1 cells. One possible explanation of this is that the 170 kDa kinase gene is physically linked to the MDR1 gene and that it has been co-amplified in the cells along with the endogenous MDR1 gene during selection for resistance. Another possibility is that selective pressure over extended periods of time has resulted in cells that have increased 170 kDa activity in addition to the amplified or over-expressed P-glycoprotein and that both these characteristics contribute to the multidrug resistance phenotype through separate mechanistic pathways.

While we show no causative connection between the 170 kDa kinase and multidrug resistance, it is of great interest that this kinase is elevated in several multidrug resistance cell lines. Studies are underway to determine at what point in the selection for multidrug resistance the apparent increase in 170 kDa kinase activity arises. We also have no definitive evidence of the cellular substrate for this kinase. It is possible that the P-glycoprotein is a cellular substrate of the 170 kDa kinase but experiments presented here do not address that possibility. Proof that this kinase is involved in P-glycoprotein mediated or non-P-glycoprotein mediated multidrug resistance will await further studies, including further biochemical characterization and isolation and transfer of the 170 kDa kinase gene to test for its effect on drug resistance.

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REFERENCES

Abraham I, Chin K-V, Gottesman MM, Mayo JK, Sampson KE (1990) Transfection of a mutant regulatory subunit gene of cAMP-dependent protein kinase causes increased drug sensitivity and decreased expression of P-glycoprotein Exp Cell Res 189 133–141

Abraham I, Hunter RJ, Sampson KE, Smith S, Gottesman MM, Mayo JK (1987) Cyclic AMP-dependent protein kinase regulates sensitivity of cells to multiple drugs Mol Cell Biol 7 3098–3106

Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases J Biol Chem 262 5592–5595

Batist G, Tulpule A, Sinha BK, Katki AG, Myers CE, Cowan KH (1986) Overexpression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells J Biol Chem 261 15544–15549

Bech-Hansen NT, Till JE, Ling V (1976) Pleiotropic phenotype of colchicine-resistant CHO cells cross-resistance and collateral sensitivity J Cell Physiol 88 23–31

Beck WT, Cirtain MC, Danks MK, Felsted RL, Safa AR, Wolverton JS, Suttle DP, Trent JM (1987) Pharmacologic, molecular and cytogenetic analysis of "atypical" multidrug resistant human leukemic cells Cancer Res 47 5455–5460

Biedler JM, Riehm H (1970) Cellular resistance to actinomycin D in Chinese hamster cells in vitro cross-resistance, radioautographic, and cytogenetic studies Cancer Res 30 1174–1184

Carlsen SA, Till JE, Ling V (1977) Modulation of drug permeability in Chinese hamster ovary cells Possible role for phosphorylation of surface glycoproteins Biochim Biophys Acta 467 238–250

Center MS (1983) Evidence that adriamycin resistance in Chinese hamster lung cells is regulated by phosphorylation of a plasma membrane glycoprotein Biochem Biophys Res Commun 115 159–166

Center MS (1985) Mechanisms regulating cell resistance to adriamycin Evidence that drug accumulation in resistant cells is modulated by phosphorylation of a plasma membrane glycoprotein Biochem Pharmacol 34 1471–1476

Chambers TC, McAvoy EM, Jacobs JW, Eilon G (1990) Protein kinase C phosphorylates P-glycoprotein in multidrug resistant human KB carcinoma cells J Biol Chem 265 7679–7686

Choi K, Chen CJ, Kriegler M, Roninson IB (1988) An altered pattern of cross-resistance in multidrug-resistant human cells results from spontaneous mutations in the mdr1 (P-glycoprotein) gene Cell 53 519–529

Cohen S (1983) Purification of the receptor for epidermal growth factor from A-431 cells Its function as a tyrosyl kinase Methods Enzymol 99 379–387

Cole SPC, Chanda ER, Dicke FP, Gerlach JH, Mirski SEL (1991) Non-p-glycoprotein-mediated multidrug resistance in a small cell lung cancer cell line evidence for decreased susceptibility to drug-induced DNA damage and reduced levels of topoisomerase II Cancer Res 51 3345–3352

Cooper JA, Hunter T (1981) Changes in protein phosphorylation in Rous sarcoma virus-transformed chicken embryo cells Mol Cell Biol 1 165–178

Elias L, Davis A (1985) Manganese-phospholipid-stimulated protein kinase activity of human leukemic cells J Biol Chem 260 7023–7028

Endicott JA, Ling V (1989) The biochemistry of P-glycoprotein-mediated multidrug resistance Annu Rev Biochem 58 137–171

Ferrell JE Jr, Martin GS (1989) Thrombin stimulates the activities on multiple previously unidentified protein kinases in platelets J Biol Chem 264 20723–20729

Ferrell JE Jr, Martin GS (1990) Identification of a 42-kilodalton phosphotyrosyl protein as a serine (threonine) protein kinase by renaturation Mol Cell Biol 10 3020–3026

Fine RL, Patel J, Chabner BA (1988) Phorbol esters induce multidrug resistance in human breast cancer cells Proc Natl Acad Sci USA 85 582–586

Goldstein LJ, Galski H, Fojo A, Willingham M, Lai S-L, Gazdar A, Pirker R, Green A, Crist W, Brodeur GM, Lieber M, Cossman J, Gottesman MM, Pastan I (1989) Expression of a multidrug resistance gene in human cancers J Natl Cancer Inst 81 116–124

Gottesman MM, Pastan I (1988) The multidrug transporter, a double-edged sword J Biol Chem 264 12163–12166

Hamada H, Hagiwara K-I, Nakajima T, Tsuruo T (1987) Phosphorylation of the M_r 170,000 to 180,000 glycoprotein specific to multidrug-resistant tumor cells effects of verapamil, trifluoperazine, and phorbol esters Cancer Res 47 2860–2865

Horio M, Gottesman MM, Pastan I (1988) ATP-dependent transport of vinblastine in vesicles from human multidrug-resistant cells Proc Natl Acad Sci USA 85 3580–3584

Kamps MP, Sefton BM (1989) Acid and base hydrolysis of phosphoproteins bound to immobilon facilitates analysis of phosphoamino acids in gel-fractionated proteins Anal Biochem 176 22–27

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4 Nature 227 680–685

Massague J (1992) Receptors for the TGF-β family Cell 69 1067–1070

McCroskey MC, Colca JR, Pearson JD (1988) Determination of [^{32}P]phosphoamino acids in protein hydrolysates by isocratic anion-exchange high performance liquid chromatography J Chrom 442 307–315

Mellado W, Horwitz SB (1987) Phosphorylation of the multidrug resistance associated glycoprotein Biochem 26 6900–6904

Meyers MB, Merluzzi VJ, Spengler BA, Biedler JL (1986) Epidermal growth factor receptor is increased in multidrug-resistant Chinese hamster and mouse tumor cells Proc Natl Acad Sci USA 83 5521–5525

Meyers MB, Shen WPV, Spengler BA, Ciccarone V, O'Brien JP, Donner DB, Furth ME, Biedler JL (1988) Increased epidermal growth factor receptor in multidrug-resistant human neuroblastoma cells J Cell Biochem 38 87–97

Morse BS, Roninson IB (1990) The role of nucleotide-binding sites in P-glycoprotein function Proceedings Amer Assoc Cancer Res 31 361

Posada JA, McKeegan EM, Worthington KG, Morin MJ, Jaken S, Tritton TR (1989) Human multidrug resistant KB cells overexpress protein kinase C involvement in drug resistance Cancer Commun 1 285–292

Riordan JR, Ling V (1985) Genetic and biochemical characterization of multidrug resistance Pharmacol Ther 28 51–75

Shabel Jr, FM, Skipper HE, Trader MW, Laster Jr, WR, Griswold Jr, DP, Corbett TH (1983) Establishment of cross-resistance profiles for new agents Cancer Treatment Reports 67 905–922

Sebolt J, Havlick M, Hamelehle K, Nelson J, Leopold W, Jackson R (1989) Activity of the pyrazoloacridines against multidrug-resistant tumor cells Cancer Chemother Pharmacol 24 219–224

Shen D-W, Cardarelli C, Hwang J, Cornwell M, Richert N, Ishii S, Pastan I, Gottesman MM (1986) Multiple drug-resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin or vinblastine show changes in expression of specific proteins J Biol Chem 261 7762–7770

Shen DW, Lu Y-G, Chin KV, Pastan I, Gottesman MM (1991) Human hepatocellular carcinoma cell lines exhibit multidrug resistance unrelated to MDR1 gene expression J Cell Science 98 317–322

Staats J, Marquardt D, Center MS (1990) Characterization of a membrane-associated protein kinase of multidrug-resistant HL60 cells which phosphorylates P-glycoprotein J Biol Chem 265 4084–4090

Waterfield MD, Mayes EL, Stroobant P, Bennet PLP, Young S, Goodfellow PN, Banting GS, Ozanne B (1982) A monoclonal antibody to the human epidermal growth factor receptor J Cell Biochem 20 149–161

Yu G, Ahmad S, Aquino A, Fairchild CR, Trepel JB, Ohno S, Suzuki K, Tsuruo T, Cowan KH, Glazer RI (1991) Transfection with protein kinase C-alpha confers increased multidrug resistance to MCF-7 cells expressing P-glycoprotein Cancer Comm 3 181–189

Zijlstra JG, de Vries EGE, Mulder NH (1987) Multifactorial drug resistance in an adriamycin-resistant human small cell lung carcinoma cell line Cancer Res 47 1780–1784